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TITLE: Reinnervation of Paralyzed Muscle by Nerve-Muscle-Endplate Band Grafting

PRINCIPAL INVESTIGATOR: Liancai Mu, M.D., Ph.D.,

RECIPIENT: Hackensack University Medical Center,
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Fort Detrick, Maryland 21702-5012

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Hackensack, NJ 07601-1914
USA

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this research is to promote the efficacy of our recently developed new surgical technique called nerve-muscle-endplate band grafting (NMEG) for muscle reinnervation in a rat model. We modified the NMEG procedure by implanting the NMEG pedicle from a donor muscle into the native motor zone (NMZ) in the experimentally denervated muscle. Three months after surgery, functional recovery of the paralyzed muscles treated with the NMEG-NMZ technique was evaluated by muscle force measurement. The extent of nerve regeneration in the target muscle was assessed using immunohistochemical techniques. Our primary findings showed that NMEG-NMZ technique resulted in better functional recovery (82% of the control) as compared with our original NMEG procedure (67%). The NMEG-NMZ induced extensive nerve regeneration in the treated muscle. These results suggest that native motor zone in the skeletal muscle is an ideal site for muscle reinnervation. NMEG-NMZ technique appears to have the potential for the treatment of muscle paralysis.

15. SUBJECT TERMS Peripheral nerve injury, muscle reinnervation, nerve-muscle-endplate band grafting, nerve regeneration, motor endplate band, native motor zone, muscle force, functional recovery

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Peripheral nerve injury is very common in both military and civil circumstances. There is a pressing need to seek novel approaches for restoration of paralyzed muscles as the presently used methods result in poor functional recovery. We developed a new reinnervation technique called nerve-muscle-endplate band grafting (NMEG) in the rat model. The concept is that a healthy motor endplate band with a nerve branch and terminals that innervate an expendable muscle can be transplanted into a more functionally important denervated muscle for restoring its motor function. Our previous studies funded by NIH demonstrated that the NMEG technique resulted in better functional recovery (67% of control) as compared with standard nerve end-to-end anastomosis (55% of control). The NMEG technique appears to be a technical advance; however, to be clinically useful the extent of functional reinnervation needs to be improved. The purpose of the proposed work funded by DOD is to augment the efficacy of this technique by creating an ideal environment that physically facilitates axon-endplate connections and biologically enhances nerve regeneration. Specifically, the surgical procedure is modified by transplanting an NMEG pedicle from the sternohyoid (SH) donor muscle into the native motor zone (NMZ) within the denervated sternomastoid (SM) recipient muscle, instead of an endplate-free area in the SM as designed in the original NMEG procedure. The effectiveness of the NMEG-NMZ (NN) technique in functional reinnervation is investigated in a rat model. In addition, intraoperative 1-hour of electrical stimulation (ES) and focal administration of exogenous neurotrophic factors (ENF) are also used to determine their beneficial effects on nerve regeneration, muscle reinnervation, and functional recovery. In this research, two reinnervation models (immediate and delayed) are used. Immediate reinnervation is performed immediately after SM nerve transaction, while delayed reinnervation is carried out at the end of 3 months after SM nerve transaction. The animals are randomly assigned to 10 groups (15 rats/each group), 8 NN-related treatment groups and 2 technique control groups (immediate and delayed direct nerve implantation, DNI). The animals undergo post-operative evaluations after a 3-month recovery period. Multiple techniques are used to determine the effects of NMEG-NMZ and adjunctive treatments (i.e., ES and ENF) on functional recovery and muscle reinnervation.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Peripheral nerve injury, muscle reinnervation, nerve-muscle-endplate band grafting, nerve regeneration, motor endplate band, native motor zone, muscle force, functional recovery

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals and objectives of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Goal 1: To evaluate functional recovery of the paralyzed muscles treated by the NMEG-NMZ technique with/without ES and ENF.

Goal 2: To determine the extent of neural regeneration and axon-endplate connections in the treated muscles.

Goal 3: To document histological and immunohistochemical alterations in the treated muscles.

Milestones:
- Surgical procedures for immediate reinnervation will be accomplished in year 1 and those for delayed reinnervation will be completed in year 2.
- Electrophysiological studies in Goal 1 will be accomplished in year 2.
- The timetable for conducting the studies on neural and muscular tissues in Goals 2 and 3 is in years 3 & 4.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

• **Major Activities:**

In the past year, we have performed the following major activities.

Animal surgeries: We have performed 120 operations on 60 animals (2 operations for each rats) in 4 immediate reinnervation groups (15 rats/per group) that include: 1) Imm-NMEG/native motor zone (Imm-NN) surgery; 2) Imm-direct nerve implantation (Imm-DNI) technique control procedure; 3) Imm-NN plus injection of a mixture of nerve growth factor (NGF) and FGF-2 (Imm-NN/ENF); and 4) Imm-NN plus 1-hour electrical stimulation (Imm-NN/ES). All the surgical procedures were successfully performed.

Muscle force measurements: The animals in the above 4 groups were subjected to muscle force measurement 3 months after surgery to evaluate functional recovery. The force data from animals in Imm-NN and Imm-DNI groups have been analyzed and the results are summarized below (see Key Outcomes). The force data from the animals in Imm-NN/ENF and Imm-NN/ES groups have been collected and will be analyzed.

Tissue studies: At the end of physiological experiments, the right experimental SM and left control for each animal were removed, measured, and prepared for tissue studies. The results from the animals in Imm-NN and Imm-DNI groups have been analyzed and summarized below. The muscle samples from the rats in Imm-NN/ENF and Imm-NN/ES groups will be processed and analyzed in the following year.

Paper writing: We have submitted two scientific papers for publication (*see Appendices*).

• **Specific Objectives:**

Data from the animals in Imm-NMEG-NMZ (Imm-NN) and Imm-DNI groups have been analyzed to determine the differences in the outcomes between both techniques.

Objective 1: To determine the degree of functional recovery of the paralyzed muscles reinnervated by either NMEG-NMZ technique or DNI-NMZ method.

Objective 2: To determine the extent of nerve regeneration in the muscles reinnervated by NMEG-NMZ and DNI-NMZ procedures.

Objective 3: To document morphological and histological changes in the NMEG-NMZ and DNI-NMZ reinnervated muscles.

• **Major Procedures**

Data from the rats in Imm-NN experimental group (immediate NMEG-native motor zone transplantation) and Imm-DNI (immediate direct nerve implantation) technique control group have been collected, analyzed, and summarized as follows.

NMEG-NMZ surgical procedure

The surgical procedures for NMEG-NMZ technique have been described in our paper (*Neurosurgery, under review, see Appendices*). Briefly, the right SM muscle was denervated by resecting its innervating nerve. The native motor zone (NMZ) of the right SM was outlined and a muscular defect (recipient bed) with the same dimensions as the NMEG (**Figs 1 & 2**) was made in the NMZ of the denervated SM muscle. An NMEG pedicle was harvested from the right SH donor muscle. An NMEG contained a block of muscle (~6 x 6 x 3 mm), axon terminals, and numerous MEPs. The well-prepared NMEG was embedded in the SM muscle defect and sutured. Thus, the experimentally denervated SM muscle was immediately reinnervated with NMEG-NMZ technique (**Figs 1 & 2**). Surgical procedures were successfully performed for each rat.

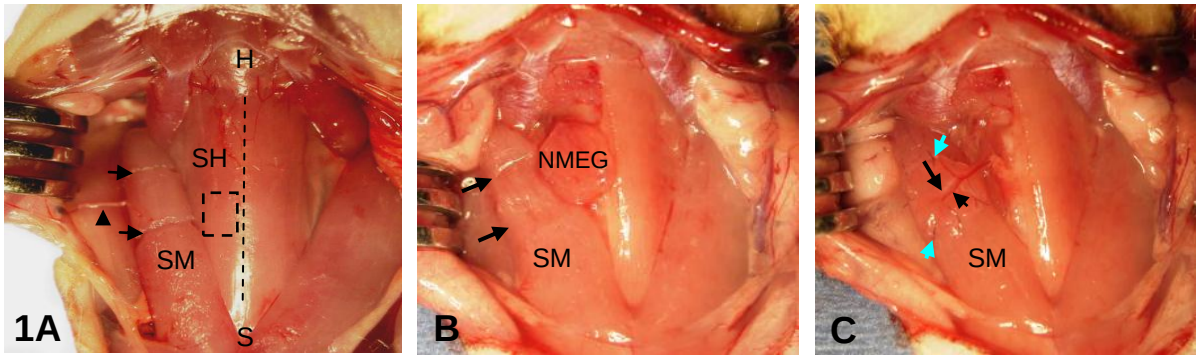


Fig. 1. Photographs from a rat, showing surgical procedures for NMEG-NMZ reinnervation technique. **A:** surgically outlined motor zones in the recipient SM (between fiber cuts as indicated by arrows) and in the donor SH (boxed region). The arrowhead indicates the SM nerve. The dashed line indicates the midline between both SH muscles. H, hyoid bone; S, sternum. **B:** an NMEG pedicle was harvested from the ipsilateral SH muscle and a muscular defect (recipient bed) of the same dimensions as the NMEG (between arrows) was made in the denervated motor zone of the SM muscle. **C:** The prepared NMEG was embedded in the recipient bed and sutured with 10-0 nylon microsutures (green arrows). Note that the implanted NMEG contained a nerve branch (large black arrow) and blood vessels (small black arrow).

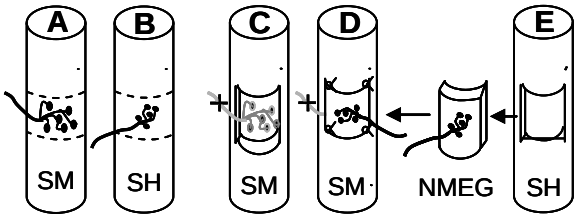


Fig. 2. Schematic illustrations of NMEG-NMZ technique. Normal SM (**A**) and SH (**B**) muscles, showing the native motor zone (NMZ) (outlined region). **C:** The SM muscle is denervated by transecting its nerve and a muscular defect is made in the NMZ of the SM. **D:** The denervated SM muscle is reinnervated by implanting an NMEG pedicle from the SH muscle (**E**) into the muscular defect in the SM.

DNI-NMZ procedure

The surgical procedures for NMEG-NMZ technique have been described in our paper (*Journal of Surgical Research, under review, see Appendices*). Briefly, the right SM muscle was denervated by transecting its innervating nerve at its entrance to the muscle (motor point). The proximal stump of the severed SM nerve was immediately buried into a small muscle slit made in the motor zone of the denervated SM muscle, and secured in position with an epineurial suture of 10-0 nylon (**Fig. 3**).

Muscle force measurement

Three months after surgery, the degree of functional recovery of the reinnervated and contralateral SM muscles in the Imm-NN and Imm-DNI groups were examined using muscle force measurement. The details regarding muscle force measurement have been given in our paper (*Journal of Surgical Research, under review, see Appendices*). Briefly, the distal tendon of the SM muscle was severed, tied with 2-0 silk suture, and connected to a servomotor lever arm with a force transducer. The nerve branch supplying the NMEG was stimulated. Isometric contractions of the SM were obtained with 200 ms trains of biphasic rectangular pulses. The duration of each phase of stimulation pulse was set at 0.2 ms and train frequency was set at 200 pulses/s. The stimulation current was gradually increased until the tetanic force reached a plateau (**Fig. 4**).

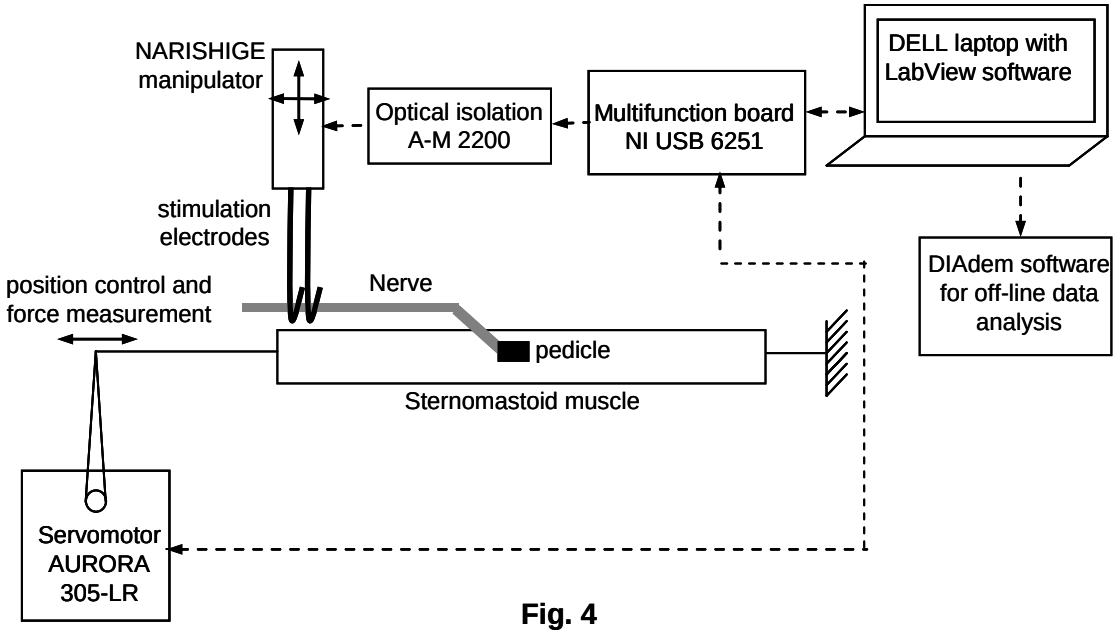


Fig. 4

Tissue studies

Muscle samples from the rats in the IMM-NN and Imm-DNI groups have been sectioned and stained using various histological and immunohistochemical methods. Cross sections from the rostral or caudal portion of the SM muscle were used for routine hematoxylin and eosin staining to examine alterations in the muscle structure and myofiber morphology (*Neurosurgery, under review, see Appendices*). Some sagittal sections from the middle SM containing the NMEG implant were immunostained with neurofilament staining and double staining to show regenerating axons and MEP reinnervation. The density of the regenerating axons on a stained section was measured with public domain ImageJ software (v. 1.45s; NIH, Bethesda, Maryland).

• **Key Outcomes**

Functional recovery

The functional outcome of reinnervation was evaluated by muscle force measurement. The percentage of functional recovery of the reinnervated SM muscle was determined as compared with that of the contralateral control muscle in each rat. Postoperative evaluations demonstrated that NMEG-NMZ technique resulted in very good functional recovery. Specifically, at 3 months after surgery, the mean muscle force of the NMEG-NMZ reinnervated muscles was measured to be 82% of the control **(Fig. 5**), whereas that of the DNI-NMZ reinnervated muscles was 64% of the control. The results demonstrated that the NMEG-NMZ technique resulted in better functional recovery as compared with our originally designed NMEG procedure (67% of the control) and DNI-NMZ (64%) (*Neurosurgery and Journal of Surgical Research, under review, see Appendices*). Clearly, NMEG-NMZ technique has unique advantages over original NMEG procedure and DNI-NMZ method

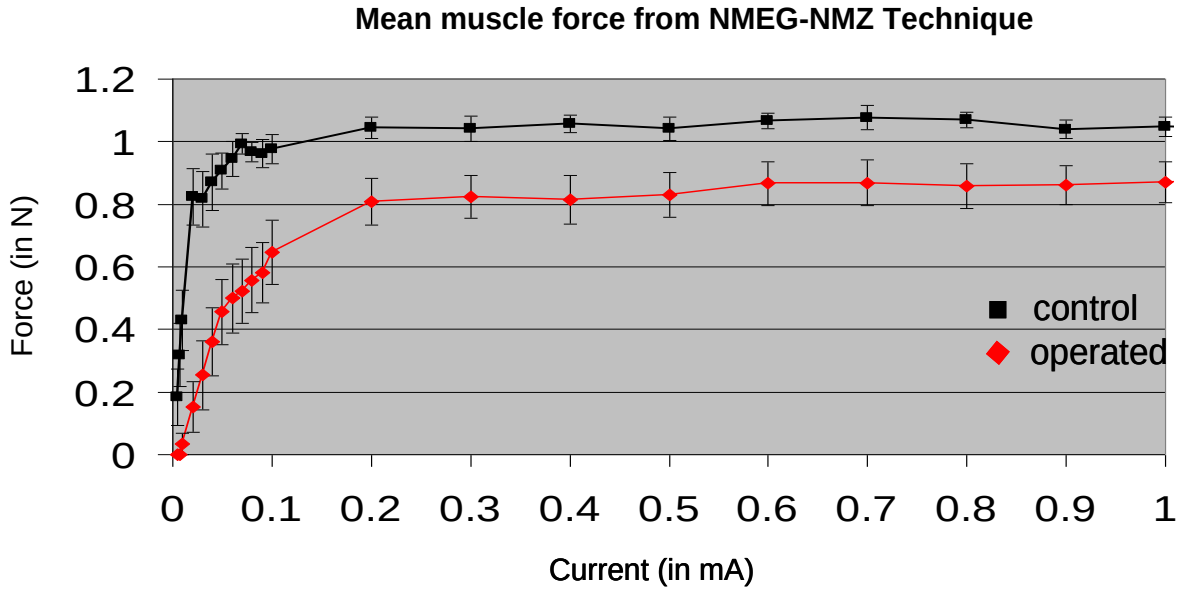


Fig. 5. Mean muscle force from operated and control SM muscles of the rats in the Imm-NN group. The passive tension was set at a moderate level (0.08 N). Stimulation was made with a 0.2-second train of 0.2-millisecond-wide biphasic pulses at frequency of 200 Hz. The lower graph (B) shows in expanded scale the early portion of the upper graph (A). Operated SM muscle with implanted NMEG pedicle (shown in red) when compared to control muscle at the opposite side (shown in black) has larger stimulation threshold, reach the level of maximal force with larger stimulation current and has smaller maximal force. Maximal muscle force level was calculated as the average muscle force to stimulation currents from 0.6-1mA. Average maximal muscle force level at the operated side (0.865 N) was 81.6% of muscle force at the control side (1.060 N). Vertical bars represent the standard deviation of the mean.

Muscle weight and myofiber morphology

At the end of experiment, both SM muscles of each rat were removed and weighed. In Imm-NN group, gross appearance and size of the NMEG-NMZ reinnervated SM muscles (**Fig. 6A**) were similar to those of the contralateral unoperated muscles (**Fig. 6B**). The NMEG-NMZ reinnervated SM muscles were larger in size (89% of the control) than the DNI reinnervated muscles (71% of the control) and 3-month-denervated SM (44% of the control) muscles (**Fig. 6C**). These findings indicate that NMEG-NMZ reinnervated muscles had slight muscle atrophy.

Hematoxylin and eosin-stained cross muscle sections showed that the NMEG-NMZ reinnervated SM muscles exhibited very good preservation of muscle structure and myofiber morphology with less fiber atrophy and connective tissue hyperplasia as compared with the control and denervated muscles (**Fig. 6A'-C'**) (*Neurosurgery and Journal of Surgical Research, under review, see Appendices*).

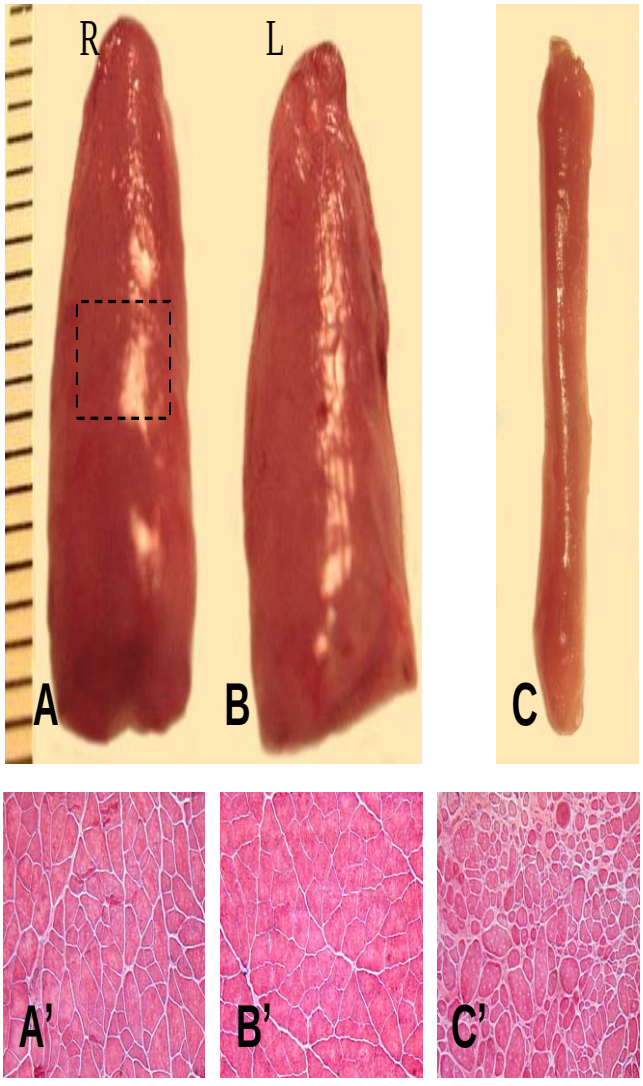


Fig. 6. Gross appearance, muscle mass, and myofiber morphology of the NMEG-NMZ reinnervated, normal, and denervated sternomastoid (SM) muscles in the rats. **A-B,** a pair of SM muscles removed from a rat 3 months after NMEG-NMZ surgery. Note that the mass of the right (R) reinnervated SM muscle (**A**) was close to that of the left (L) control muscle (**B**). The outlined region in the right SM is the location of the transplant. **C,** A 3-month completely denervated SM muscle. Note that the denervated SM showed a more significant loss of muscle mass as compared with the reinnervated and normal SM muscles. **A'-C',** hematoxylin and eosin-stained cross sections from the SM muscles in **A-C**. Note that the reinnervated SM (**A'**) exhibited very good preservation of muscle structure and myofiber morphology with less fiber atrophy as compared with the normal (**B'**) and denervated (**C'**) muscles. The SM denervated for 3 months exhibited significant myofiber atrophy. x 200 for A'-C'.

Nerve regeneration and muscle reinnervation

Three months after muscle reinnervation with NMEG-NMZ and DNI-NMZ methods, muscle sections immunostained for NF showed that NMEG-NMZ technique resulted in extensive nerve regeneration in the target muscle. The data are being collected and analyzed. **Figure 7** gives an example to show the regenerating axons in the NMEG-NMZ reinnervated and contralateral control muscles in a rat. Note that the regenerating axons from the implanted NMEG supply the denervated native motor zone within the target muscle. Moderate level of regenerating axons was found in the DNI-NMZ reinnervated muscles (*data shown in paper 2, see Appendices*). The density of the regenerating axons in the reinnervated muscles was indicated by the number and the area fraction of NF-ir axons. The mean number and area of the NF-ir axons in the DNI-NMZ reinnervated SM muscles was computed to be 62% and 51% of the contralateral control muscles, respectively (see *paper 2 in Appendices*). In summary, both reinnervation methods induce nerve regeneration in the native motor zone of the target muscles. NMEG-NMZ results in better nerve regeneration and muscle reinnervation as compared with DNI-NMZ method. These findings are consistent with force data. Specifically, NMEG-NMZ technique resulted in better functional recovery (82% of the control) as compared with DNI-NMZ method (64% of the control).

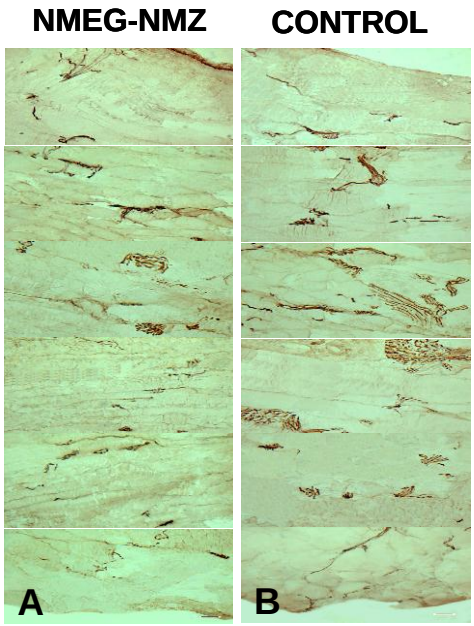


Fig. 7. Comparison of the densities of intramuscular nerve fascicles and axons between right sternomastoid (SM) muscle reinnervated with NMEG-NMZ (**A**) and left control muscle (**B**) in a rat. Photomicrographs were taken from sagittal section (40-µm thick) from the motor zone in each muscle. The sections were immunostained with antibody against neurofilament (NF) showing nerve fascicles and axons in the operated and control muscles. The images were taken from the ventral surface (top) to the dorsal surface (bottom) of each muscle. Note that regenerating axons in the NMEG-NMZ reinnervated SM muscle (**A**) are distributed throughout the muscle in the native motor zone of the treated muscle. Scale bar in B = 100 µm.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The following studies will be performed during the next reporting period.

• Animals in the Imm-NN/ES/ENF group (n=15 rats) will undergo SM nerve transection, 1-hour electrical stimulation of the SH nerve supplying the pedicle, NMEG implantation, and injection of a mixture of nerve growth factor (NGF) and FGF-2 into the in the middle region of the treated SM.

• We will analyze the collected force data from the SM muscles (n=30) of the rats in the Imm-NN/ENF group (n=15 rats).

• The muscle samples (n=60) obtained from the animals in the Imm-NN/ENF groups (n=15 rats) will be subjected to sectioning and staining using various histochemical and immunohistochemical techniques to assess nerve regeneration after surgeries.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our primary findings showed that the modified NMEG technique (i.e., NMEG-NMZ) resulted in better functional recovery (82% of the control) as compared with our original NMEG procedure (67%). The NMEG-NMZ induced extensive nerve regeneration in the treated muscle. These results suggest that native motor zone in the skeletal muscle is an ideal site for muscle reinnervation. Our on-going studies focus on further promoting the efficacy of the NMEG-NMZ technique by using either nerve stimulation or nerve growth factors to enhance nerve regeneration. We hope these studies will provide useful data for future research directions to improve the effectiveness of the NMEG-NMZ technique. We believe that NMEG-NMZ will become a useful method in the near future to treat our patients with muscle paralysis.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Two papers resulting from the work under this award have been submitted to scientific journals for publication. 1) Mu L, Sobotka S, Chen J, Nyirenda T. Modified nerve-muscle-endplate band grafting technique for muscle reinnervation. I. Surgical procedures and functional outcomes. Neurosurgery (under review), 2015. Acknowledgement of federal support (yes). 2) Sobotka S, Chen J, Nyirenda T, Mu L. Outcomes of muscle reinnervation with direct nerve implantation into the native motor zone of the target muscle. Journal of Surgical Research (under review), 2015. Acknowledgement of federal support (yes).

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

* We participated in a conference and presented part of our data from this research. The title was "Modified nerve-muscle-endplate band grafting technique for muscle reinnervation" by Sobotka S, Chen J, Nyirenda T, and Mu L. Presented at the Gorge Perez Research Colloquium at the Seton Hall University, South Orange, NJ, April 24, 2015.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Liancai Mu	no change
Stanislaw Sobotka	no change
Jingming Chen	no change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report." If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.
QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

A quad chart has been updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

In Appendices, the following two papers have been submitted to scientific journals for publication.

1) Mu L, Sobotka S, Chen J, Nyirenda T. Modified nerve-muscle-endplate band grafting technique for muscle reinnervation. I. Surgical procedures and functional outcomes. Neurosurgery (under review), 2015. Acknowledgement of federal support (yes).

2) Sobotka S, Chen J, Nyirenda T, Mu L. Outcomes of muscle reinnervation with direct nerve implantation into the native motor zone of the target muscle. Journal of Surgical Research (under review), 2015. Acknowledgement of federal support (yes).

Reinnervation of Paralyzed Muscle by Nerve-Muscle-Endplate Band Grafting

ERMS Number 12223004
W81XWH-14-1-0442

PI: Liancai Mu, MD, PhD **Org:** Hackensack University Medical Center **Award Amount:** $800,000

Study/Product Aim(s)

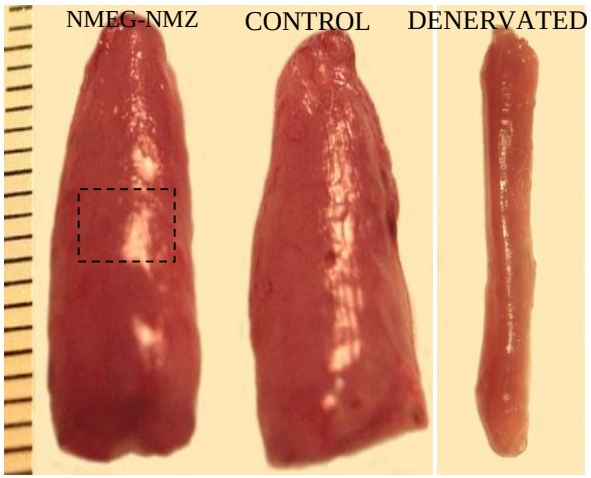
Aim 1: To evaluate functional recovery of the paralyzed muscles treated by NMEG-NMZ technique with/without ES and ENF.

Aim 2: To determine the extent of neural regeneration and axon-endplate connections in the treated muscles.

Aim 3: To document histological and immunohistochemical alterations in the treated muscles.

Approach

- Microsurgical procedures (NMEG-NMZ and DNI techniques).
- Intra-operative 1-hour electrical stimulation (ES).
- Focal administration of exogenous NGF and FGF-2.
- EMG and muscle force measurements.
- Various staining methods to label regenerating axons, MEPs.
- Analyze muscle fiber types and myosin heavy chains.



NMEG-NMZ reinnervated, control, and denervated SM muscles.

Timeline and Cost

Activities CY	1	2	3	4
Surgery				
Physiological evaluations				
Neural studies				
Muscle studies & data analyses				
Estimated Budget ($K)	**$200k**	**$200k**	**$200k**	**$200k**

Updated: (10/10/2015)

Goals/Milestones

CY1 Goal – Microsurgery,functional evaluations, and neural studies.
☐ Perform surgeries, muscle force measurement, and nerve staining.
CY2 Goal – Microsurgery, functional evaluations, and tissue studies.
☐Complete surgeries and perform physiological and tissue studies.
CY3 Goal – Functional evaluation and tissue studies.
☐ Complete physiological testing and perform tissue studies.
CY4 Goal – Tissue studies, data collection, and data analyses.
☐ Investigate nerve regeneration and muscle fiber type and myosin heavy chain composition.

Comments/Challenges/Issues/Concerns

If timelines change, comment here.
If off by more than one quarter in spending, comment here.

Budget Expenditure to Date

Projected Expenditure: $50,000
Actual Expenditure: $40,046

Neurosurgery

Modified Nerve-Muscle-Endplate Band Grafting Technique for Muscle Reinnervation. I. Surgical Procedures and Functional Outcomes

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research - Animal
Section/Category:	Peripheral Nerve
Corresponding Author:	Liancai Mu, MD, Ph.D Hackensack University Medical Center Hackensack, NJ UNITED STATES
Order of Authors:	Liancai Mu, MD, Ph.D
	Stanislaw Sobotka, Ph.D
	Jingming Chen, MD
	Themba Nyirenda, PhD
Manuscript Region of Origin:	UNITED STATES
Abstract:	BACKGROUND: Restoration of useful function after peripheral nerve injury is a major challenge for reconstructive surgery and rehabilitation medicine. OBJECTIVE: To describe our modified nerve-muscle-endplate band grafting (NMEG) procedure and document functional recovery. METHODS: Muscle reinnervation with modified NMEG technique was studied in the rat model to test our hypothesis that optimal functional recovery could be obtained by transplanting a NMEG pedicle harvested from a neighbor healthy and undamaged donor muscle to the native motor zone (NMZ) of the target muscle. In the present study, the NMEG pedicle was harvested from the sternohyoid muscle and transplanted into the NMZ within the ipsilateral experimentally denervated sternomastoid muscle. A NMEG contained a muscle block, a nerve branch with nerve terminals, and a motor endplate band with numerous neuromuscular junctions. The extent of functional recovery was evaluated postoperatively using maximal tetanic force measurement. RESULTS: At 3 months after surgery, the mean muscle force of the reinnervated muscles was measured to be 82% of the control. The results from this study demonstrated that the NMEG-NMZ technique resulted in better functional recovery as compared to our originaly designed NMEG procedure (67% of the control), in which a NMEG was implanted into an endplate-free area in the target muscle. CONCLUSION: The NMEG-NMZ yielded excellent muscle force recovery of the reinnervated muscles. Our results suggest that the NMZ of the target muscle is the best site for NMEG implantation to obtain optimal functional outcomes.
Suggested Reviewers:	n/a n/a N/A hsu@yahoo.com We do not have suggested reviewers.
	n/a n/a Australian National University hsu@yahoo.com We do not have suggested reviewers.
Opposed Reviewers:	n/a n/a First Affiliated Hospital of Nanchang University hsu@yahoo.com We do not have oppose reviewers.
	n/a n/a n/a hsu@yahoo.com We do not have oppose reviewers.

Additional Information:	
Question	**Response**
Significance of the Work: Please include a brief statement summarizing the significance of the work and in particular how it differs from and advances existing literature.	As the currently available methods for muscle reinnervation result in poor functional recovery, we developed a new technique called “nerve-muscle-endplate band grafting (NMEG)”. An NMEG pedicle was harvested from a sternohyoid muscle (donor) and implanted into the caudal portion (endplate-free area) of ipsilateral denervated sternomastoid muscle (recipient). The NMEG pedicle was composed of an intact donor nerve branch and a block of muscle that contained nerve terminals and a motor endplate band. Our previous studies showed that NMEG resulted in better functional recovery (67% of the control) as compared with standard end-to-end nerve repair (55%). The goal of the present study was to augment the outcomes of our originally designed NMEG by changing implantation site from endplate-free area to the native motor zone (NMZ) within the target muscle. The concept is that the efficacy of the NMEG procedure could be improved by creating an ideal environment that physically facilitates neural connections between the regenerating axons from the NMEG pedicle and the denervated native endplates in the target muscle. The results demonstrated that the NMEG-NMZ technique resulted in excellent recovery of muscle force (82% of the control). These findings suggest that the NMZ of the target muscle is the best site for NMEG implantation to obtain optimal functional outcomes.
Compliance with Research Reporting Guidelines: *Neurosurgery* endorses several reporting guidelines and requires authors to submit their research articles in accordance with the appropriate guideline statement(s) and checklist(s). Completed applicable checklists and flow diagrams must be included with submissions. Research articles that must be submitted according to the appropriate reporting guideline(s) include, but are not limited to: randomized trials, systematic reviews, meta-analyses of interventions, meta-analyses of observational studies, diagnostic accuracy studies, and observational epidemiological studies (eg, case series, cohort, case-control, and cross-sectional studies). Consult the EQUATOR Network, which maintains a useful, up-to-date list of guidelines as they are published, with links to articles and checklists: http://www.equator-network.org. Please confirm below that information is reported according to the relevant reporting guideline(s) and any required materials are included with the submission:	Not Applicable - Submission Does Not Report Research That Requires Adherence to Reporting Guideline(s)
Statistical Analysis: For manuscripts that report statistics, the Editor requires that the authors provide evidence of statistical consultation or expertise. If your article includes statistics, has the information reported been evaluated by an expert?	Yes

IRB/Ethics Approval: Please indicate if your study has received institutional review board/ethics approval. If yes, these materials are readily available should the Editor request them.	Not Applicable - Not Required For This Study

September 2, 2015

Nelson M. Oyesiku, MD, PhD, FACS,
Editor-in-Chief, NEUROSURGERY
1510 Clifton Road, NE
Suite G65
Atlanta, GA 30322

Dear Dr. Oyesiku,

We would like to submit our manuscript entitled "**Modified Nerve-Muscle-Endplate Band Grafting Technique for Muscle Reinnervation. I: Surgical Procedures and Functional Outcomes**" for publication in *Neurosurgery*.

Our initially developed method entitled "Nerve-Muscle-Endplate band Grafting: A New Technique for Muscle Reinnervation" was published in *Neurosurgery* in 2011 [Neurosurgery 69(2 Suppl Operative):ons208-224, 2011]. This surgical procedure was further refined to promote its efficacy. The modified technique yielded more optimal outcomes as compared with the initially designed procedure.

The work reported in this manuscript has not been, and is not intended to be, published anywhere except in *Neurosurgery*.

Authorship:
Liancai Mu, M.D., Ph.D.* (Corresponding author)
Stanislaw Sobotka, Ph.D.,
Jingming Chen, M.D.,
Themba Nyirenda, Ph.D.,

Disclosure:
This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs under Award No. W81XWH-14-1-0442. The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

Ethical experimentation:
The experimental protocols are approved by the Institutional Animal Care and Use Committee and are in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

Thank you for your consideration.

Sincerely,

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Modified Nerve-Muscle-Endplate Band Grafting Technique for Muscle Reinnervation. I. Surgical Procedures and Functional Outcomes

Liancai Mu, M.D., Ph.D.,* Stanislaw Sobotka, Ph.D.,*‡ Jingming Chen, M.D.,* Themba Nyirenda, Ph.D.,*

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New York, N.Y. 10029

Abbreviated title: *MODIFIED NERVE-MUSCLE-ENDPLATE BAND GRAFTING TECHNIQUE*

Acknowledgement:

The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office.

This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs under Award No. W81XWH-14-1-0442 (to Dr. Liancai Mu). Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

In conducting research using animals, the investigators adhere to the laws of the United States and regulations of the Department of Agriculture. This protocol was approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of rats.

Disclosure: The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

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ABSTRACT

BACKGROUND: Restoration of useful function after peripheral nerve injury is a major challenge for reconstructive surgery and rehabilitation medicine.

OBJECTIVE: To describe our modified nerve-muscle-endplate band grafting (NMEG) procedure and document functional recovery.

METHODS: Muscle reinnervation with modified NMEG technique was studied in the rat model to test our hypothesis that optimal functional recovery could be obtained by transplanting a NMEG pedicle harvested from a neighbor healthy and undamaged donor muscle to the native motor zone (NMZ) of the target muscle. In the present study, the NMEG pedicle was harvested from the sternohyoid muscle and transplanted into the NMZ within the ipsilateral experimentally denervated sternomastoid muscle. A NMEG contained a muscle block, a nerve branch with nerve terminals, and a motor endplate band with numerous neuromuscular junctions. The extent of functional recovery was evaluated postoperatively using maximal tetanic force measurement.

RESULTS: At 3 months after surgery, the mean muscle force of the reinnervated muscles was measured to be 82% of the control. The results from this study demonstrated that the NMEG-NMZ technique resulted in better functional recovery as compared to our originaly designed NMEG procedure (67% of the control), in which a NMEG was implanted into an endplate-free area in the target muscle.

CONCLUSION: The NMEG-NMZ yielded excellent muscle force recovery of the reinnervated muscles. Our results suggest that the NMZ of the target muscle is the best site for NMEG implantation to obtain optimal functional outcomes.

KEY WORDS: Functional recovery, Muscle force measurement, Muscle paralysis, Muscle reinnervation, Nerve-muscle-endplate band grafting, Peripheral nerve injury, Sternohyoid muscle, Sternomastoid muscle

ABBREVIATIONS: MEP, motor endplate; **NMEG**, nerve-muscle-endplate band grafting; **NMZ**, native motor zone; **PNI**, peripheral nerve injury; **SH**, sternohyoid muscle; **SM**, sternomastoid muscle

INTRODUCTION

Neuromuscular denervation is a common consequence of peripheral nerve injuries (PNIs) and neurological diseases. PNI is common in both military and civil circumstances. Military PNIs are mostly caused by gunshot and blast injuries from bombs and other explosive devices.[1] Recent publications have reported the numbers of casualties and character of combat wounds and their associated management of the current conflicts in Iraq and Afghanistan.[2-4] Combat-related head/face/neck and extremity injuries account for 21-40% and 54% of all battle wounds, respectively and are the main causes for the greatest quantity of disability among combat casualties.[3,5-10] In the civilian population, PNIs are mostly caused by vehicle accidents[11,12] and surgical intervention.[13] There are 200,000 and 300,000 cases with PNIs in the United States and Europe, respectively, each year.[14,15] Muscle paralysis caused by PNI is a main source of chronic disabilities which limit the opportunities to work and diminish quality of life. The nerve repair techniques and principles for PNIs in the military population are the same as those described in the civilian population[8] and have changed little over the past decades.[16] Despite advancements in microsurgical techniques, PNIs are still a major challenge for reconstructive surgeons.

Based on various situations, PNI-induced muscle paralysis can be managed with nerve end-to-end anastomosis (EEA), end-to-side neurorrhaphy, nerve grafting, nerve transfer, muscular neurotization, tubulization techniques, and many others.[17-25] However, the currently available methods for muscle reinnervation result in poor muscle reinnervation and functional recovery. In general, EEA is commonly used when the two stumps of an injured nerve can be approximated without tension.[24,25] Unfortunately, only about 50% of patients treated with EEA nerve repair regain useful function.[18,19] Factors behind poor functional recovery include tension of the anastomosis, neuroma formation, scaring, and loss of the nerve fiber population.[26] Studies have demonstrated that in EEA fewer nerve fibers could pass through the coaptation

site and reach the target muscle.[27] When a tension-free EEA is not technically possible, end-to-side neurorrhaphy is an alternative to repair an injured nerve when the proximal nerve stump is unavailable.[28,29] The distal stump of an injured nerve is sutured to the side of an intact donor nerve. However, this procedure induces less axon regeneration and functional recovery compared to EEA.[30-33]

A significant nerve defect is a common clinical situation. At present, autologous nerve grafts, nerve transfers, and tubulization techniques with natural or artificial conduits are used for nerve-gap repair. However, nerve grafting has been associated with poor functional outcomes when there is a long distance from the level of the injury to the target muscle. The recovery rate of motor function after autogenous nerve grafting is less than 40%.[21] Tubulization techniques are feasible only in short nerve gaps. If the gap exceeds 1.0-1.5 cm, regeneration is poor.[22] Longer defects (4.0-6.0 cm) result in useful reinnervation in only 13% of cases with reconstruction of upper-extremity PNIs with conduits.[34,35] For the PNIs in which the proximal nerve stump is unavailable, nerve transfer is an option. Nerve transfer is the surgical coaptation of a healthy nerve donor to a denervated nerve. A nerve branch that innervates expendable muscles can be used to repair a functionally more important distal stump of an injured nerve. Many nerve-to-nerve transfers have been employed to repair the injured nerves in the hand and upper extremity with mixed results.[36] In many cases with trauma, especially combat-related head/neck and extremity injuries, the distal nerve stump might be not available for nerve repair. In this condition, nerve repair or nerve-grafting procedures are inapplicable because of the lack of a distal nerve stump. The only reconstructive option to reinnervate that muscle is direct nerve implantation or muscular neurotization.[37,38]

In an effort to get a better solution for the management of muscle paralysis, we have developed a new reinnervation technique called “nerve-muscle-endplate band grafting (NMEG)”.[39] Over the past 5 years, we have studied the NMEG technique by determining innervation patterns[39,40] and contractile properties[41] of the rat recipient and donor muscles, and conducted surgical feasibility studies[39] and a series of reinnervation experiments using the NMEG technique and conventional EEA in a rat model.[39,42-45] A NMEG pedicle was harvested from a sternohyoid (SH) muscle (donor) and sutured to the denuded surface of the ipsilateral

experimentally denervated sternomastoid (SM) muscle (recipient). The NMEG pedicle was composed of 3 components: a block of muscle (6x6x3 mm), an intact donor nerve branch and nerve terminals, and a motor endplate (MEP) band with numerous neuromuscular junctions. Several lines of evidence from a number of quantitative analyses demonstrated that the NMEG technique results in better muscle reinnervation and functional recovery (67% of control) as compared with standard nerve EEA method (55%).[39,42-45]

Although the NMEG technique resulted in better results than the conventional EEA, entire muscle reinnervation and complete functional recovery was not achieved.[39,43-45] We found that a few regenerating axons from the implanted NMEG pedicle reached the distal portion of the target muscle 3 months after surgery. Approximately 1/3 of the distal myofibers in the target muscle were not well reinnervated.[45] These findings suggest that partial muscle reinnervation accounts for incomplete functional recovery. We believe that implantation site of the NMEG would be a critical factor influencing outcomes. In our previous studies, a NMEG was implanted into an aneural region in the recipient muscle. In this case, regenerating axons from the NMEG pedicle may need more time to reach the most distal muscle fibers and form new MEPs. These findings stimulated us to modify the NMEG procedure by implanting the NMEG into the native motor zone (NMZ) in the target muscle where the nerve terminals were degenerated and the MEPs were denervated. We hypothesize that NMEG-NMZ procedure would facilitate rapid MEP reinnervation that prevents the target muscle from irreversible loss of the denervated MEPs.

The goal of this study is to augment the outcomes of our originally designed NMEG by changing implantation site from MEP-free area to the NMZ within the target muscle using the same animal model. Thus, the unique advantages of the NMEG-NMZ over the initial NMEG procedure could be determined by comparing the results of this study with our published data from the original NMEG experiments.[39,44,45]

MATERIALS AND METHODS

Animals

Fifteen female 3-month-old Sprague-Dawley rats (Taconic Laboratories, Cranbury, NJ) with initial body masses ranging from 200 to 250 g were used in this study. The animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee. All animals were handled in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The animals were kept in a 22°C environment in light-dark cycles (12/12h) with free access to food and water. The rats were individually housed in standard cages in the state-of-the-art animal housing facilities of Hackensack University Medical Center.

Surgical Procedures for NMEG-NMZ Technique

In this study, sternohyoid (SH) and sternomastoid (SM) muscles were chosen to serve as a donor and recipient, respectively, because they were used in our previous studies.[39-45] Therefore, there is a solid database regarding their morphological features, innervation patterns, and contractile properties available for comparison.

Microsurgical procedures were conducted under aseptic conditions. All animals underwent general anesthesia by intraperitoneal administration of a mixture of ketamine (80 mg/kg body weight) and xylazine (5 mg/kg body weight). With the rat supine, a midline cervical incision was made extending from the hyoid bone to the sternum to expose the right SM and SH muscles and their innervating nerves. With the aid of an Olympus SZX12 Stereo zoom surgical microscope (Olympus America Inc, Center Valley, Pennsylvania), the exposed muscles and nerves were further prepared for NMEG-NMZ transplantation.

First of all, the right SM muscle was denervated by resecting a 5-mm segment of its innervating nerve. The cut ends of the nerve were then coagulated with a bipolar cautery to prevent nerve regeneration. Then, the motor zone in the muscle was outlined according to the motor point (the entry point of the motor branch into the muscle) (Figure 1). A muscular defect (recipient bed) with the same dimensions as the NMEG pedicle (see below) was made in the motor zone in the middle portion of the right denervated SM muscle (Figures 2 and 3).

Next, an NMEG pedicle was harvested from the right SH donor muscle. The details regarding the preparation for harvesting an NMEG have been given in our previous publication.[39] Briefly, the SH nerve branch was identified on the lateral margin of the middle portion of the muscle and traced from the motor point to the motor zone where axon terminals and an MEP band are located. An NMEG containing a block of muscle (approximately 6 x 6 x 3 mm), axon terminals, and an MEP band was outlined and harvested from the SH muscle in continuity with its pedicle of motor nerve branch and feeding vessels (Figures 2 and 3). A functioning NMEG was confirmed by observing its twitch contractions on nerve stimulation. Before implantation, the superficial muscle fibers on the ventral aspect of the NMEG pedicle were removed to create a denuded surface for better neural regeneration.

Finally, the well-prepared NMEG was embedded in the SM muscle defect and sutured with four to six 10-0 nylon microsutures (Figures 2 and 3). Thus, the experimentally denervated SM muscle was immediately reinnervated with NMEG-NMZ technique.

After surgery, the wound was closed in layers with interrupted simple sutures of 4-0 Prolene. The animals had to be awake before being returned to the animal colony and began a 3-month period of postoperative recovery.

Postoperative Evaluations

At the end of the 3-month recovery period, the efficacy of the NMEG-NMZ technique for muscle reinnervation was evaluated functionally and histologically.

Determining the Degree of Functional Recovery

Maximal tetanic force measurement was used to evaluate the functionality of the muscles treated by NMEG-NMZ technique. Muscle force measurement is a useful approach to assess the mechanical function and contractile properties of a muscle.[46-48] Our studies[39,42-45] and others[49-51]used force measurement to detect the degree of functional recovery of the reinnervated cervical strap muscles.

The details regarding the force measurement of the rat SM muscle have been given in our publications[39,41-45] Briefly, the right reinnervated and the left control SM muscles in each animal were exposed and dissected free from the surrounding tissues. The rostral tendon of each muscle was severed close to the insertion, tied with a 2-0 suture, and attached to a servomotor lever arm (model 305B Dual-Mode Lever Arm System; Aurora Scientific Inc, Aurora, Ontario, Canada). Muscle force of the right reinnervated SM was measured by stimulating the SH nerve branch supplying the NMEG, whereas that of the left control muscle was measured by stimulating the intact SM nerve. Each of the nerves was identified, isolated, and draped over a bipolar stimulating electrode for nerve stimulation.

Trains of biphasic rectangular pulses of different current were delivered to the stimulated nerve using our stimulation and recording system. The system is built based on a multifunction National Instruments Acquisition Board (National Instruments Corp, Austin, Texas) and is controlled by user-written LabVIEW software (National Instruments Corp). The detailed description of this acquisition system was presented in our previous paper.[41] Briefly, the system consists of an NI USB board (6251, 16 bit 1.25 Ms/s, National Instruments, Austin, TX) connected to a DELL laptop with a custom written program using National Instruments' labVIEW 8.2 software. The system generates stimulation pulses, which after isolation from the ground through an optical isolation unit (Analog Stimulus Isolator Model 2200, A-M Systems, Inc., Carlsborg, WA) are used for the current controlled nerve stimulation.

Isometric contraction of the SM muscle was produced with 200-millisecond trains of biphasic rectangular pulses. The duration of each phase of stimulation pulse was set at 0.2 milliseconds and the train frequency was set at 200 pulses per second. The stimulation current was gradually increased until the tetanic force reached a plateau. A break of at least 1 minute was taken between two stimulations. The maximum value of muscle force during the 200-millisecond contraction was identified, as well as initial passive tension before stimulation. The difference between the maximal active force and the preloaded passive force was used as the muscle force measurement. The force generated by the contraction of the SM muscle was transduced with the servomotor of a 305B lever system and displayed on a computer screen. At the moment

of force measurement, the lever arm was stationary, and the muscle was adjusted to the optimal length for the development of maximum force.

During the experiment, the animal was placed supine on a heating pad (homoeothermic blanket system; Stoelting, Wood Dale, Illinois). The core body temperature was monitored with a rectal thermistor and maintained at 36ºC. The muscle and nerve examined were bathed regularly with warmed mineral oil throughout the testing to maintain muscle temperature between 35ºC and 36ºC.

The force data were obtained and processed by an acquisition system built from a multifunction I/O National Instruments Acquisition Board (NI USB 6251; 16 bit, 1.25 Ms/s; National Instruments) connected to a Dell laptop with a custom-written program using LabVIEW 8.2 software. The system produced stimulation pulses, which, after isolation from the ground through an optical isolation unit (Analog Stimulus Isolator model 2200; AM Systems, Inc, Carlsborg, Washington), were used for the current controlled nerve stimulation. The acquisition system was also used to control muscle length and to collect a muscle force signal from the 305B Dual-Mode Lever System. Collected data were analyzed offline with DIAdem 11.0 software (National Instruments).

Examining Implanted NMEG

At the end of the experiments, SM muscles on both sides in each animal were removed, weighed, and photographed. Three NMEG-NMZ reinnervated right SM muscles were processed with Sihler's stain, a wholemount nerve mapping technique to see if the NMEG was precisely implanted into the motor zone of the target muscle.

Sihler's Stain. The details regarding Sihler's stain have been given in our previous publications.[39,52] Briefly, the removed muscles were fixed for 3 weeks in 10% unneutralized formalin; macerated for 2 weeks in 3% aqueous potassium hydroxide (KOH) solution; decalcified for 1 week in Sihler solution I (one volume glacial acetic acid, one volume glycerin, and six volumes 1% w/v aqueous chloral hydrate); stained for 3 weeks in Sihler solution II (one volume stock Ehrlich's hematoxylin, one volume glycerin, and six volumes

1% w/v aqueous chloral hydrate); destained for 3 hours in Sihler solution I; immersed for 1 hour in 0.05% w/v lithium carbonate solution to darken the nerves; cleared for 3 days in 50% v/v aqueous glycerin; and preserved for 4 weeks in 100% glycerin with a few thymol crystals for transparency. After transillumination by a xenon light source (model 610; Karl Storz, Endoscopy-America, Culver City, California), the stained muscles were dissected and photographed.

Examining Muscle Fiber Morphology

The SM muscles from twelve rats were frozen in melting isopentane cooled with dry ice and cut on a cryostat (Reichert-Jung 1800; Mannheim, Germany) at –25ºC. Some sections were stained with routine hematoxylin and eosin (H&E) staining to examine alterations in the muscle structure and myofiber morphology, whereas others were prepared for different purposes.

Data Analysis

Wet weights and force values of the operated and unoperated SM muscles in each rat were computed. Both variables of the reinnervated SM muscles were expressed as the percentages of the values of the contralateral control muscles. All data were reported as mean ± SD. The Student *t* test (paired, 2 tailed) was used to compare differences in the mean muscle force and mean muscle weight between operated and unoperated SM muscles. A difference was considered statistically significant at $P < 0.05$.

RESULTS

Maximal Tetanic Muscle Force

Maximum tetanic tension in the NMEG-NMZ reinnervated SM and the contralateral control muscles was evaluated at the end of the 3-month recovery period. The averaged force values for the treated and control muscles were summarized in Figure 4. For each rat, the percentage of functional recovery of the SM muscle reinnervated with NMEG-NMZ technique was determined compared with that of the contralateral

control muscle. Our previous studies have demonstrated that optimal muscle length could be achieved by stretching the muscle with moderate tension of 0.08 N.[39,41] Electric stimulation of the nerve branch innervating the NMEG at low intensity (0.02-0.0 mA) produced visible muscle contraction. A lower (0.0075-0.01 mA) stimulation threshold was obtained when the nerve innervating the left intact SM muscle was stimulated. An increase in the stimulation current resulted in an increase of muscle force until it reached horizontal asymptote. During nerve stimulation on the operated side, this saturation level was reached with 0.2 mA. The saturation level was reached at smaller current (0.1 mA) on the control side. The reinnervated SM showed an average maximum tetanic force of 0.87 ± 0.23 N (control, 1.06 ± 0.10 N; $P < 0.005$). The reinnervated SM muscles produced 82% of the maximal tetanic tension of the contralateral control muscles.

Implanted NMEG

Our previous studies have demonstrated the nerve supply patterns and the locations of the MEP bands in the SH donor muscle and the SM recipient muscle in the rat.[39,40] These data were useful for accurately harvesting an NMEG from the SH muscle and precisely implanting the NMEG into the native motor zone within the experimentally denervated SM muscle.

Sihler's stain showed that microsurgery for NMEG-NMZ muscle reinnervation was successfully performed as indicated by precise implantation of the NMEG into the motor zone within the target SM muscle (Figure 5A). Each of the implanted NMEG contained a SH nerve branch and numerous nerve terminals. In our original NMEG procedure, the NMEG was implanted into the caudal portion of the SM muscle (Figure 5B).

Muscle Weight and Myofiber Morphology

In each rat, gross appearance and size of the operated SM was similar to those of the contralateral unoperated muscle. The reinnervated SM muscles were greater in size than the SM muscles with complete denervation for 3 months (Figure 6A-C). The mean value and standard deviation of the wet weight was 0.332

± 0.047 g in the group of reinnervated SM muscles, whereas 0.374 ± 0.044 g in the group of contralateral control muscles. The differences in muscle weights in both groups were relatively small but consistent across all animals and therefore statistically significant (P <0.0001). Specifically, the reinnervated SM muscles weighed 89% of the weight of contralateral control muscles (Table). These findings indicate that NMEG-NMZ reinnervated muscles had slight muscle atrophy.

Hematoxylin and eosin-stained cross muscle sections showed that the NMEG-NMZ reinnervated SM muscles exhibited very good preservation of muscle structure and myofiber morphology with less fiber atrophy and connective tissue hyperplasia as compared with the control and denervated muscles (Figure 6A'-C').

DISCUSSION

Summary

The present experimental study aimed at augmenting the efficacy of our originally designed NMEG microsurgical procedure for muscle reinnervation. The results from this study demonstrated that the NMEG-NMZ technique resulted in more optimal recovery of muscle force (82% of the control) as compared with the originally designed NMEG procedure (67% of the control).[39,44,45] The mean value of the weight of the NMEG-NMZ reinnervated SM muscles was 89% of the contralateral control muscles. These findings indicate that the NMEG-NMZ technique greatly enhanced the efficacy of our initial NMEG procedure.

Concept and Rationale of the NMEG-NMZ Reinnervation Technique

As the currently available reinnervation methods result in poor nerve regeneration and functional recovery, we developed NMEG technique. The development of the NMEG technique is based on the concept that a paralyzed muscle could be reinnervated by transplanting an NMEG from a neighboring donor muscle. A healthy MEP band with a nerve branch and terminals that innervates an expendable muscle can be transplanted to a more functionally important denervated muscle for restoring its motor function. A

transplanted NMEG could provide an abundant source of nerve terminals and MEPs for nerve regeneration and muscle reinnervation to restore motor function of the target muscle.

Our original NMEG procedure yielded better functional recovery (67% of the controls) than the conventional nerve end-to-end anastomosis (55%).[39,42-45] The encouraging results can be attributed to its unique advantages. First, NMEG provides an abundant source of nerve terminals and MEPs for nerve regeneration and muscle reinnervation. Second, NMEG leads to minimal nerve fiber injury and degenerative reaction. The transected axons in the cut margins and the denuded surface of the NMEG are much more distal, thereby retaining a greater proportion of the donor nerve fibers and developing minimal nerve degeneration. The transected axons in the NMEG are considerably separate from each other in 3-dimensional space compared with the density of axons in a peripheral nerve. Finally, NMEG has sufficient pedicle-recipient muscle interfaces, which provide enough space for axonal regeneration. The axons could start to regenerate at multiple points in the implanted NMEG and grow across the pedicle-recipient muscle interfaces to reach the recipient muscle fibers.

Although our original NMEG procedure resulted in better outcomes as compared with nerve end-to-end anastomosis, entire muscle reinnervation and complete functional recovery have not been achieved.[39,44,45] We found that approximately 1/3 of the distal myofibers in the target muscle were reinnervated only by a small number of regenerating axons.[45] Therefore, incomplete functional recovery following NMEG surgery is associated with partial muscle reinnervation.

The present study showed that implantation site of the NMEG is a critical factor influencing outcomes. In our previous experiments, an NMEG was implanted into an aneural region in the caudal portion of the recipient muscle. In this case, regenerating axons from the NMEG pedicle may need more time to reach the most distal muscle fibers and form new MEPs. These findings stimulated us to modify the NMEG procedure. We hypothesized that the efficacy of our NMEG procedure may be improved by creating an ideal environment that physically facilitates neural connections between the regenerating axons from the NMEG

pedicle and the denervated native MEPs in the recipient muscle. To test this hypothesis, we implanted the NMEG into the native motor zoon (NMZ) in the target muscle as described in this study.

The development of the NMEG-NMZ technique (band-to-band procedure) is based on the concept that denervated MEPs in the NMZ of the target muscle are preferential sites for reinnervation. One reason for the impaired target reinnervation could be degradation of MEPs during prolonged denervation. NMEG-NMZ procedure would facilitate rapid MEP reinnervation to avoid irreversible loss of the denervated MEPs in the target muscle. The importance of nerve regeneration onto the sites of the original MEPs is highlighted by various animal experiments. Recent studies[53] have shown that poor motor recovery after peripheral nerve injury resulted from a failure of synapse reformation because of the delay in motor axons reaching their target. Barbour and colleagues (2012)[54] emphasized that *"Functional motor recovery after peripheral nerve injury is predominantly determined by the time to motor endplate reinnervation and the absolute number of regenerated motor axons that reach target."* MEP regions of mammalian muscle fibers are preferentially reinnervated as a consequence of some special property of the muscle fiber at this site. The MEP is a highly specialized structure, optimized for the rapid transmission of information from the presynaptic nerve terminal to the post-synaptic muscle fiber. It serves to efficiently communicate the electrical impulse from the motor neuron to the skeletal muscle to signal contraction. Studies have demonstrated that after nerve injury regenerating axons preferentially form synapses at original synaptic sites.[55-59] As reported, 30 days after nerve transection all the regenerating nerve fibers grew into and innervated the regions of the original MEPs.[55] The MEPs may exert an attraction on the regenerating axons. Synaptic basal lamina at the MEP contains molecules that direct the formation of synaptic specializations on regenerating axon terminals and myofibers.[60] Some other chemotropic substance released from the MEPs may attract the regenerating axons in the vicinity. Using direct nerve implantation model, some investigators observed preferential reinnervation of the native MEPs in the target muscle by abundant regenerating axons and sprouts.[61,62]

The potential advantages of the NMEG-NMZ technique over the original NMEG procedure are that nerve regeneration distances are shortened and rapid axon-MEP connections can be established. The optimal

outcomes of the NMEG-NMZ reinnervation may be attributed to the fact that the regenerating axons from the NMEG could rapidly reinnervate the denervated MEPs and form functional synapses.

Research Directions

Current methods of nerve repair yield suboptimal outcomes and new methods and technologies to enhance target reinnervation are needed in the clinic. Optimal muscle reinnervation requires regenerating axons to reinnervate the target muscle in a timely fashion to avoid irreversible loss of target MEPs by degeneration and fibrosis.[63] This study showed that the NMEG-NMZ resulted in optimal outcomes for immediate muscle reinnervation. Further work is underway to document its potential for delayed muscle reinnervation.

Our ongoing studies are to investigate the neural basis for successful restoration of muscle function following NMEG-NMZ surgery. We believe that the outcomes of the NMEG-NMZ technique would be improved by using additional approaches that accelerate neural regeneration. Among the strategies used for promoting axon regeneration, electrical stimulation has been shown to enhance nerve regeneration and muscle reinnervation. For example, Gordon and colleagues[64-66] developed a very brief 1-hour period of low-frequency (20 Hz) continuous electrical stimulation of the transected and repaired proximal nerve stump at the time of nerve repair. Their studies have shown that this method has the potential to accelerate axon regeneration and improve functional outcomes. Another approach for promoting nerve regeneration would be the administration of exogenous neurotrophic factors such as nerve growth factor (NGF), basic fibroblast growth factor (FGF-2), and others which have been demonstrated to enhance axon regeneration.[67,68] Further investigations into the potential use of these approaches for muscle reinnervation are warranted. Our ongoing studies are to determine the beneficial effects of 1-hour electrical stimulation and focal administration of exogenous neurotrophic factors on the NMEG-NMZ reinnervated muscles. It is important to document whether a combination of the NMEG-NMZ and intraoperative brief electrical stimulation and/or focal administration of exogenous neurotrophic factors would enhance functional recovery more than either

treatment alone. We hypothesize that the efficacy of the NMEG-NMZ reinnervation technique may be further improved by creating an ideal environment that biologically accelerates neural regeneration. Such studies would lay a firm foundation for the ultimate clinical application of this new reinnervation technique.

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FIGURE LEGENDS

FIGURE 1. *Outlining motor zones containing nerve terminals and motor endplate (MEP) band within the recipient sternomastoid (SM) and donor sternohyoid (SH) muscles of the rat.* **A-C,** *stained and fresh right SM muscles showing the motor zone within the muscle.* **A,** *a Sihler's stained SM showing the intramuscular nerve branching and terminals.* **B,** *a wholemount acetylcholinesterase (AChE) stained SM muscle localizing the motor endplate (MEP) band (arrow).* **C,** *a fresh SM muscle illustrating the motor zone outlined during surgery (between fiber cuts on the surface of the muscle as indicated by arrows). Note that a single motor nerve branch (arrowheads in* **A** and **C***) enters into the SM in the middle portion of the muscle and then gives off many twigs and numerous nerve terminals. The motor zone within the SM muscle contains numerous intramuscular nerve terminals (***A***) and a MEP band with numerous neuromuscular junctions (arrow in* **B***).* **D-F,** *stained and fresh right SH muscles of the rat showing the motor zone within the muscle.* **D,** *a Sihler's stained SH.* **E,** *a wholemount AChE stained SH muscle localizing the MEP band (arrow).* **F,** *a fresh SM muscle illustrating the motor zone outlined during surgery (between fiber cuts on the surface of the muscle as indicated by arrows). The motor zone of the SH muscle is also located in the middle of the muscle. Note that a single motor nerve branch (arrowheads in* **D** and **F***) enters into the SM in the middle portion of the muscle and then gives off many twigs and terminals.* **G,** *a photograph from a rat during surgery, showing surgically outlined motor zones in the recipient SM (between fiber cuts as indicated by arrows) and in the donor SH (boxed region). The arrowhead indicates the SM nerve. The dashed line indicates the midline between both SH muscles. H, hyoid bone; S, sternum.*

FIGURE 2. *Photographs from a rat during surgery, showing procedures for muscle reinnervation with NMEG-NMZ technique.* **A,** *this image shows the anatomic locations of the donor (sternohyoid, SH) and recipient (sternomastoid, SM) muscles and their motor zones (enclosed regions). The SM nerve (arrow) is visualized to enter the motor zone of the SM at the lateral margin in the middle portion of the muscle. The dashed line indicates the midline between both SH muscles. H, hyoid bone; L, left; R, right; S, sternum.* **B,**

the entry point of the SM nerve (arrow) into the muscle is identified by retracting the muscle medially. **C,** *an NMEG pedicle was harvested from the ipsilateral SH muscle and a muscular defect (recipient bed) of the same dimensions as the NMEG (between arrows) was made in the denervated motor zone of the SM muscle. The superficial muscle fibers on the ventral aspect of the NMEG pedicle were removed to create a denuded surface before implantation.* **D,** *the prepared NMEG was embedded in the recipient bed and sutured with 10-0 nylon microsutures (green arrows). Note that the implanted NMEG contained a nerve branch (large black arrow) and blood vessels (small black arrow).* **E,** *this photograph shows that the SM nerve (see* **A** *and* **B***) was removed (black arrow). The green arrows indicate the microsutures.* **F,** *3 months after NMEG-NMZ transplantation, the rat neck was re-opened for muscle force measurement. Note that the implanted NMEG was healed well. The microsutures (green arrows) around the implanted NMEG pedicle as well as the nerve branch (large black arrow) and vessels (small black arrow) supplying the NMEG pedicle were still visible under an operating microscope.*

FIGURE 3. *Schematic illustrations of the procedures for muscle reinnervation with NMEG-NMZ technique. Normal sternomastoid (SM;* **A***) and sternohyoid (SH;* **B***) muscles, showing the native motor zone (outlined region) in the middle portion of each muscle.* **C,** *the SM muscle is denervated by transecting its innervating nerve and a muscular defect of the same dimensions as the NMEG pedicle is made in the native motor zone of the SM.* **D,** *the denervated SM muscle is reinnervated by implanting an NMEG pedicle harvested from the SH muscle (***E***) into the muscular defect created in the native motor zone of the target muscle.*

FIGURE 4. *Muscle force as a function of stimulation current in operated and control SM muscles. The passive tension was set at a moderate level (0.08 N). Stimulation was made with a 0.2-second train of 0.2-millisecond-wide biphasic pulses at frequency of 200 Hz. The lower graph (***B***) shows in expanded scale the early portion of the upper graph (***A***). Operated SM muscle with implanted NMEG pedicle (shown in red) when compared to control muscle at the opposite side (shown in black) has larger stimulation threshold,*

reach the level of maximal force with larger stimulation current and has smaller maximal force. Maximal muscle force level was calculated as the average muscle force to stimulation currents from 0.6-1mA. Average maximal muscle force level at the operated side (0.865 N) was 81.6% of muscle force at the control side (1.060 N). Vertical bars represent the standard deviation of the mean.

FIGURE 5. *Comparison of the implantation sites of the NMEG between NMEG-NMZ technique (***A***) and our originally designed NMEG procedure (***B***) in the rat.* **A,** *a Sihler's stained right sternomastoid (SM) muscle reinnervated with the NMEG-NMZ technique. Note that the NMEG pedicle containing a nerve branch to the sternohyoid (SH) muscle (blue arrow) and intramuscular nerve terminals was implanted into the motor zone (outlined region) of the SM in the middle portion of the muscle. Green arrow indicates a microsuture surrounding the implant.* **B,** *a Sihler's stained right SM muscle reinnervated with our originally designed NMEG procedure. Note that the NMEG pedicle outlined by microsutures (green arrow) was implanted into the caudal portion of the target SM muscle. The implanted NMEG contained a SH nerve branch (blue arrow) and nerve terminals. Black arrow indicates the SM nerve branch which enters into its motor zone (outlined region) and gives off nerve terminals.*

FIGURE 6. *Comparison of the gross appearance, muscle mass, and myofiber morphology between the NMEG-NMZ reinnervated, normal, and denervated sternomastoid (SM) muscles in the rats.* **A-B,** *a pair of SM muscles removed from a rat 3 months after surgery. Note that the mass of the right (R) reinnervated SM muscle (***A***) was close to that of the left (L) control muscle (***B***). The outlined region in the right SM is the location of the transplant.* **C,** a SM muscle denervated by transecting its nerve for 3 months. Note that the denervated SM showed a more significant loss of muscle mass as compared with the reinnervated and normal SM muscles. **A'-C',** *hematoxylin and eosin-stained cross sections from the SM muscles in* **A-C**. *Note that 3 months after surgery the NMEG-NMZ reinnervated SM (***A'***) exhibited very good preservation of muscle structure and myofiber morphology with less fiber atrophy as compared with the normal (***B'***) and denervated*

*(**C'**) muscles. The SM denervated for 3 months exhibited significant myofiber atrophy. Initial magnification x 200 for A' through C'.*

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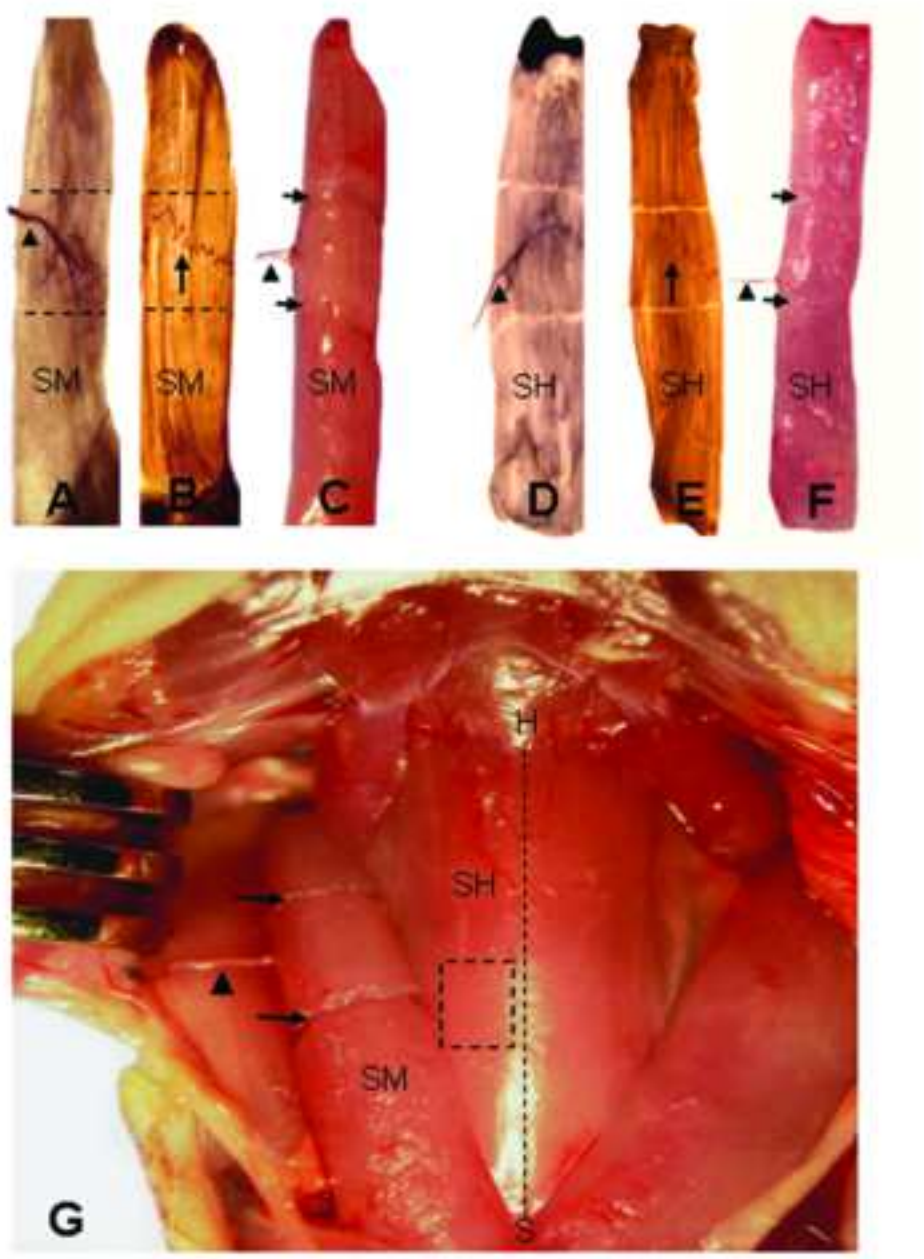

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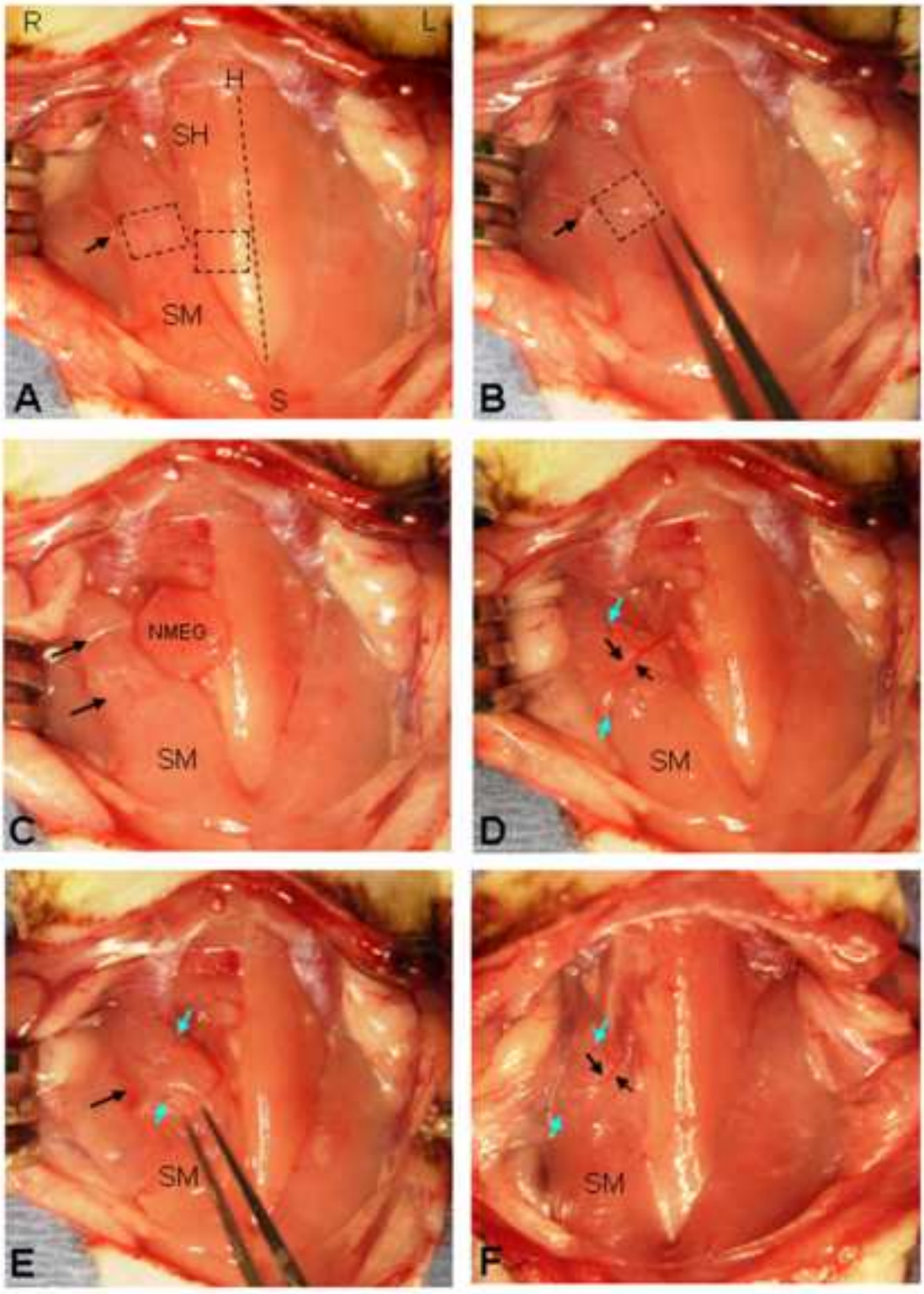

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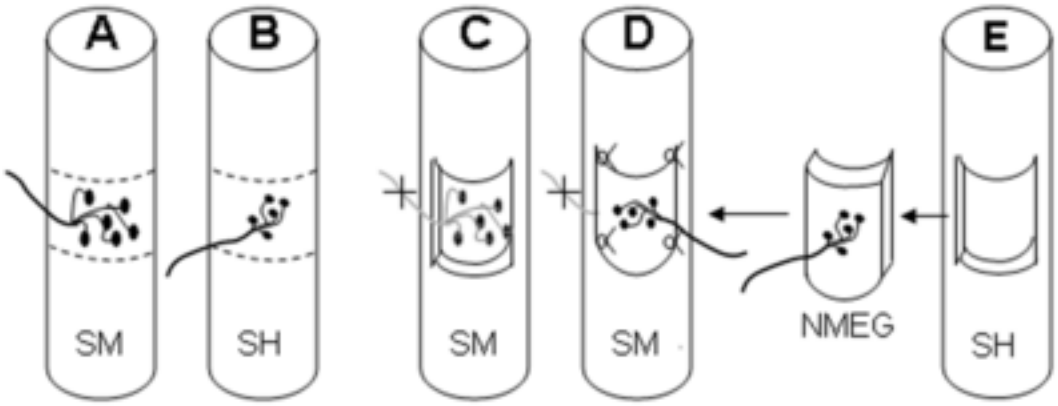

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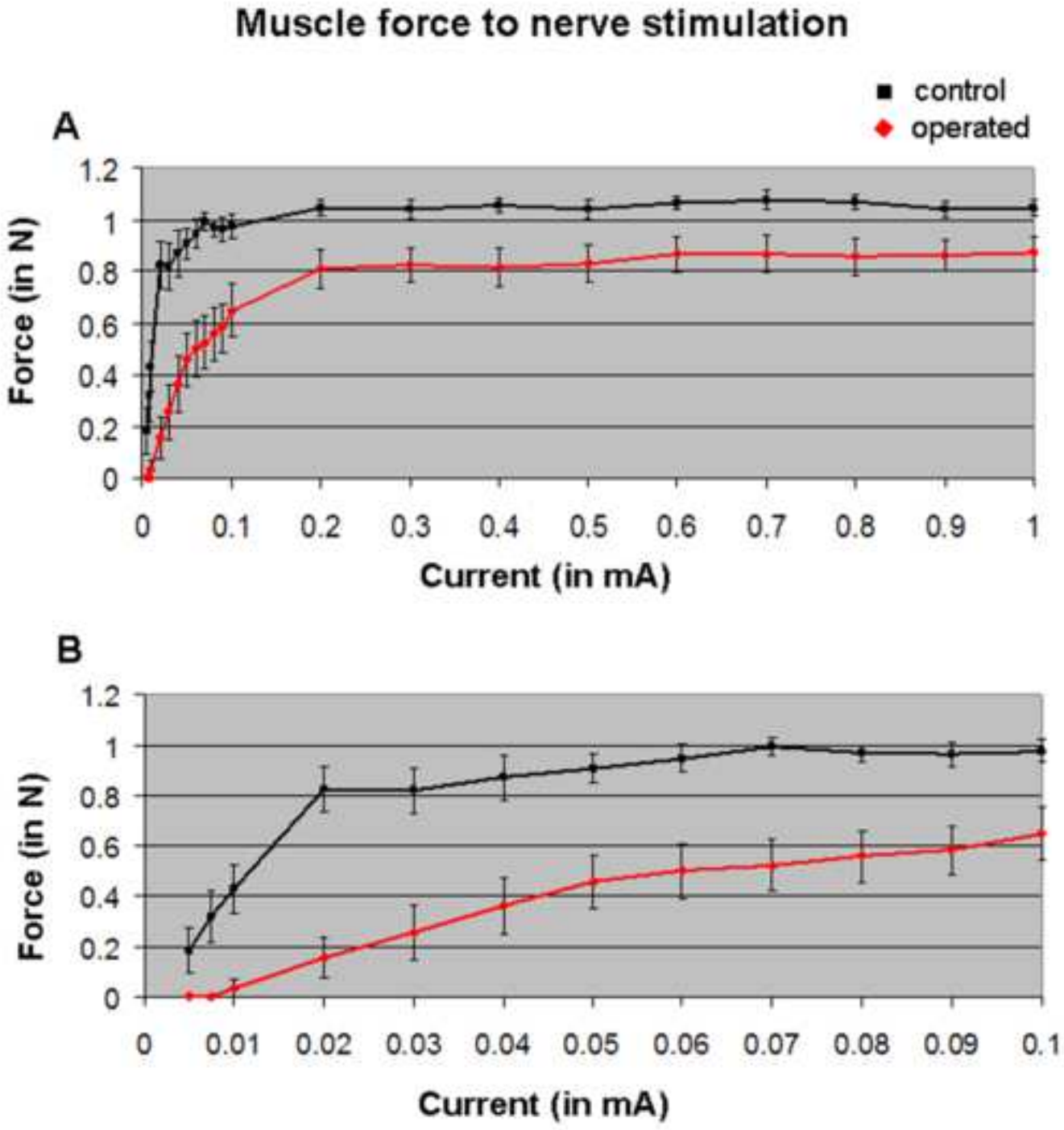

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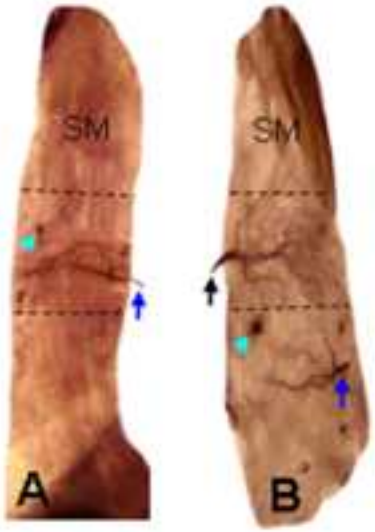

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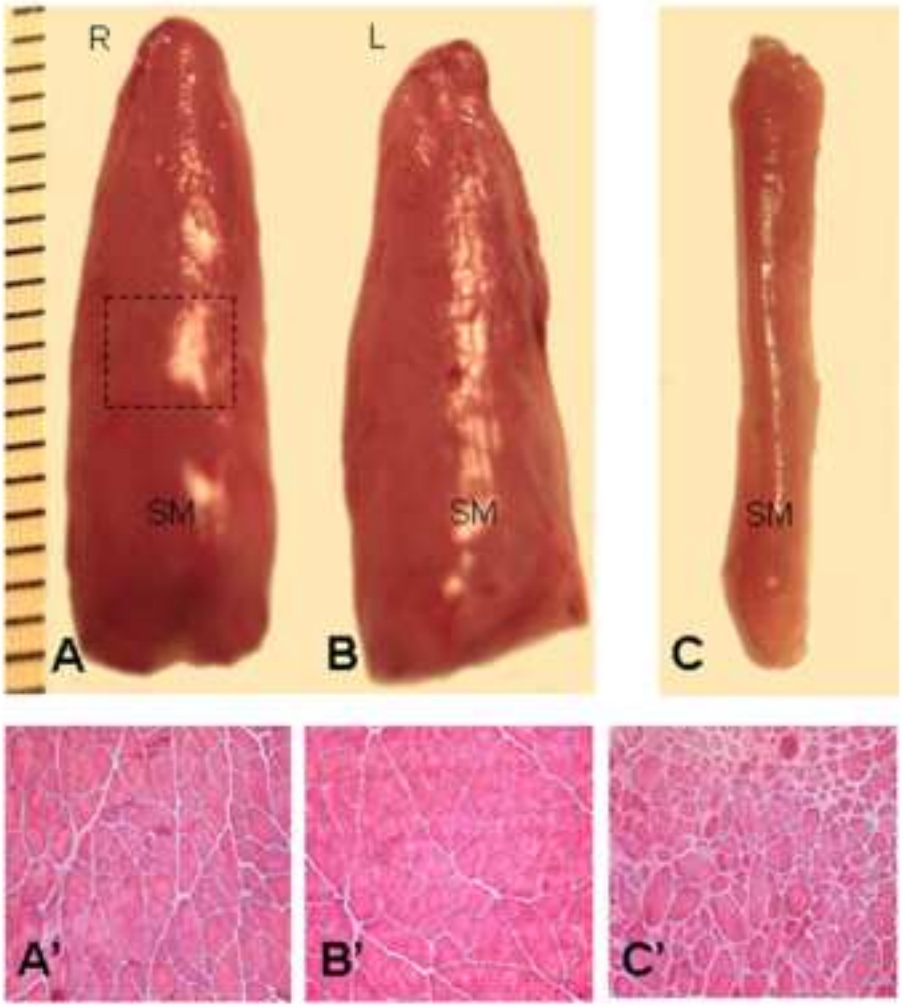

Table

TABLE. Wet Muscle Weight Measurement for the Reinnervated and Control sternomastoid (SM) Muscles in Rats

Animal No.	Body Weight, g	Right Reinnervated SM Muscle, g	Left Normal SM Muscle, g	Ratio R/L
1	333	0.309	0.333	0.928
2	328	0.332	0.397	0.836
3	319	0.318	0.357	0.891
4	285	0.287	0.328	0.875
5	386	0.245	0.316	0.775
6	373	0.373	0.407	0.916
7	281	0.320	0.343	0.933
8	330	0.396	0.437	0.906
9	329	0.305	0.327	0.933
10	355	0.335	0.358	0.936
11	380	0.341	0.392	0.870
12	304	0.326	0.338	0.964
13	358	0.433	0.462	0.937
14	315	0.373	0.410	0.910
15	328	0.290	0.398	0.729
Average	333.6	0.332	0.374	0.889
STDEV	31.8	0.047	0.044	0.065

R, right; L, left.

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Keywords: Direct nerve implantation; Peripheral nerve injury; Muscle paralysis; Muscle reinnervation; Motor endplates; Native motor zone; Nerve regeneration; Muscle force measurement

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Dr. D. W. McFadden, MD
Editor of the Journal of Surgical Research
University of Connecticut, Farmington, CT

S.A. LeMaire, MD
Editor of the Journal of Surgical Research
Baylor College of Medicine, Houston, Texas, USA

Dear Drs. McFadden and LeMaire,

We would like to submit our manuscript entitled *"Outcomes of muscle reinnervation with direct nerve implantation into the native motor zone of the target muscle" by S. Sobotka, J. Chen, T. Nyirenda, and L. Mu,* for publication in the Journal of Surgical Research.

This manuscript describes our research on reinnervation of the rat sternomastoid muscle with our novel and promising reinnervation technique using direct nerve implantation into the native motor zone of the target muscle. We show the extend of anatomical and functional recovery of this muscle 3 months after reinnervation with this technique.

Enclosed please find the manuscript with 5 Figures.

Thank you for your consideration.
Sincerely,

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Outcomes of muscle reinnervation with direct nerve implantation into the native motor zone of the target muscle

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Author's Contributions:

SS and LM contributed to the conception, design of experiments and data analysis. SS, LM and JC did the muscle force data acquisition. LM and JC used histological histochemical and immunohistochemical methods to document nerve regeneration. TN participated in statistical analysis. SS and LM wrote the article.

ABSTRACT

Background: Our recent work has demonstrated that the native motor zone (NMZ) within a given skeletal muscle is the best site for muscle reinnervation. This study was designed to investigate the efficacy of direct nerve implantation (DNI) into the NMZ of denervated sternomastoid (SM) muscle in a rat model.

Materials and methods: The right SM muscle was experimentally denervated by transecting its innervating nerve at its entrance to the muscle (motor point). The proximal stump of the severed SM nerve was immediately implanted into a small muscle slit made in the NMZ of the muscle where denervated motor endplates (MEPs) were concentrated. The outcomes of DNI-NMZ reinnervation were evaluated 3 months after surgery. Specifically, the degree of functional recovery was examined with muscle force measurement and the extent of nerve regeneration and muscle reinnervation was assessed using morphological, histochemical and immunohistochemical methods.

Results: The results showed that DNI-NMZ resulted in satisfactory recovery of muscle force (64% of the control) 3 months after surgery. Immunostained muscle sections showed that this reinnervation technique yielded abundant regenerating axons in the target muscle.

Conclusions: These findings suggest that DNI-NMZ holds promise in the treatment of muscle paralysis. Further investigations into the potential use of this technique for muscle reinnervation are warranted.

Keywords: Direct nerve implantation; Peripheral nerve injury; Muscle paralysis; Muscle reinnervation; Motor endplates; Native motor zone; Nerve regeneration; Muscle force measurement

1. Introduction

Peripheral nerve injuries (PNIs) are very common in both military and civil circumstances. In the former, PNIs are related to gunshot and blast injuries from bombs and other explosive devices [1]. In the latter, PNIs are caused primarily by vehicle accidents [2,3] and surgical intervention [4]. Current nerve repair methods include nerve end-to-end anastomosis (EEA), end-to-side neurorrhaphy, nerve grafting, nerve transfer, muscular neurotization, tubulization techniques, and others [5-13]. Unfortunately, these methods result in poor muscle reinnervation and functional recovery. Approximately 50% of patients treated with these methods fail to regain useful function [6,7,12,13]. Clearly, there is a great need to seek new approaches for restoration of paralyzed muscles as the currently available surgical treatment options lead to suboptimal clinical outcomes.

When the nerve is injured near its entrance to the muscle, nerve repair cannot be performed because the distal stump of the injured nerve is not available. In this condition, the reconstructive option to reinnervate that muscle is direct nerve implantation (DNI) or muscular neurotization [11,14]. In neurotization, the proximal stump of the original nerve or a healthy but less valuable foreign motor nerve is implanted into the target muscle to reinnervate a more important motor territory that has lost its innervation through irreparable damage to its nerve. DNI has been used for selective reinnervation of paralyzed laryngeal and facial muscles [15,16] as well as the extremities when the distal nerve stump is not available for nerve repair [11,14]. However, further studies are needed to determine its potential for muscle reinnervation and neural basis of functional recovery.

Our recent study [17] has demonstrated that the native motor zone (NMZ) within the recipient muscle is the best site for muscle reinnervation. The concept is that the NMZ in a muscle contains numerous motor endplates which are preferentially reinnervated. Previous studies showed that after nerve injury regenerating nerve fibers preferentially grow into and reinnervate the regions of the original endplates [18-22]. Using DNI model, some investigators observed preferential reinnervation of the native endplates in the target muscle by

abundant regenerating axons and sprouts [23,24]. However, little is known whether DNI-NMZ reinnervation results in satisfactory functional recovery.

This study was designed to test our hypothesis that better outcomes could be achieved by implanting a nerve stump into the NMZ within the recipient muscle. The reinnervated muscles were assessed using morphological, immunohistochemical, and electrophysiological techniques to determine the extent of muscle reinnervation and functional recovery.

2. Materials and methods

2.1. Animals

Animal experiments were performed on 3-month-old female Sprague-Dawley rats (Taconic Laboratories, Cranbury, NJ) with body masses ranging from 200 to 250 g at initial operation. The experiments and procedures were ethically reviewed and approved by the Institutional Animal Care and Use Committee prior to the onset of experiments. All animals were handled in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). The animals were housed at a constant temperature (22°C) on a 12 hour light-dark cycle and were provided with food and water in the state of the art animal housing facilities of Hackensack University Medical Center.

2.2. DNI-NMZ procedures

A total of 15 animals were used to perform DNI-NMZ procedure. Sternomastoid (SM) muscle was experimentally denervated and reinnervated with DNI-NMZ. As SM muscle model was used in our previous muscle reinnervation studies [25-31], there is a solid database regarding its innervation pattern and contractile properties available for comparison.

Surgical procedures were conducted under aseptic conditions. Animals underwent general anesthesia with a mixture of ketamine (80 mg/kg body wt) and xylazine (5 mg/kg body wt) administered

intraperitoneally. A midline cervical incision was made extending from the hyoid bone to the sternum to expose the right SM muscle and its innervating nerve under an Olympus SZX12 Stereo zoom surgical microscope (Olympus America Inc, Center Valley, Pennsylvania).

The exposed right SM and its nerve were further prepared for muscle denervation and reinnervation using DNI-NMZ technique. Briefly, the right SM muscle was denervated by transecting its innervating nerve at its entrance to the muscle (motor point). The proximal stump of the severed SM nerve was immediately buried into a small muscle slit made in the motor zone of the denervated SM muscle, and secured in position with an epineurial suture of 10-0 nylon (Fig. 1). After surgery, the wound was closed.

At the end of the 3-month recovery period, all experimental animals underwent postoperative evaluations to assess surgical outcomes of the DNI-NMZ, including functional recovery, nerve regeneration, and muscle reinnervation using electrophysiological, histological and immunohistochemical techniques.

2.3. Measurement of maximal tetanic force

Our previous studies [25,28-31] and others [16,32,33] used force measurement to detect the degree of functional recovery of the reinnervated cervical strap muscles. The details regarding the force measurement of the rat SM muscle have been given in our publications [25,28-31]. Briefly, the right reinnervated SM muscle in each animal was exposed and dissected free from the surrounding tissue without interrupting its innervating nerve. The rostral tendon of the muscle was severed close to the insertion, tied with a 2-0 suture, and connected to a servomotor lever arm (model 305B Dual-Mode Lever Arm System; Aurora Scientific Inc, Aurora, Ontario, Canada). The implanted SM nerve branch was identified, isolated, and placed on a bipolar stimulating electrode for nerve stimulation. On the control side, muscle force of the left normal SM was measured by stimulating the intact SM nerve.

A stimulation and recording system (National Instruments Corp, Austin, Texas) controlled by user-written LabVIEW software (National Instruments Corp) was used to deliver biphasic rectangular pulses to the nerve stimulated (Fig. 2). Isometric contraction of the SM muscle was produced with 200-millisecond

trains of biphasic rectangular pulses. The duration of each phase of stimulation pulse was set at 0.2 milliseconds and the train frequency was set at 200 pulses per second. The stimulation current was gradually increased until the tetanic force reached a plateau. A break of at least 1 minute was taken before the next measurement was attempted. The maximum value of muscle force during the 200-millisecond contraction was identified, as well as initial passive tension before stimulation. The difference between the maximal active force and the preloaded passive force was used as the muscle force measurement. The force generated by the contraction of the SM muscle was transduced with the servomotor of a 305B lever system and displayed on a computer screen. At the moment of force measurement, the lever arm was stationary, and the muscle was adjusted to the optimal length for the development of maximum force. For each rat, the nonoperated left SM muscle was also assessed using the same method to obtain the control value. During the experiment, the rat was placed supine on a heating pad (homoeothermic blanket system; Stoelting, Wood Dale, Illinois), and the core body temperature was monitored with a rectal thermistor and maintained at 36ºC. The muscle and nerve examined were bathed regularly with warmed mineral oil throughout the testing to maintain muscle temperature between 35ºC and 36ºC.

The force data were obtained and processed by an acquisition system built from a multifunction I/O National Instruments Acquisition Board (NI USB 6251; 16 bit, 1.25 Ms/s; National Instruments) connected to a Dell laptop with a custom-written program using LabVIEW 8.2 software. The system produced stimulation pulses, which, after isolation from the ground through an optical isolation unit (Analog Stimulus Isolator model 2200; AM Systems, Inc, Carlsborg, Washington), were used for the current controlled nerve stimulation. The acquisition system was also used to control muscle length and to collect a muscle force signal from the 305B Dual-Mode Lever System. Collected data were analyzed offline with DIAdem 11.0 software (National Instruments).

2.4. Examination of muscle structure and myofiber morphology

At the end of the experiments, SM muscles on both sides in each animal were removed and weighed. Each muscle was divided into three segments: rostral, middle, and caudal. The muscle segments were frozen in melting isopentane cooled with dry ice, in a cryostat (Reichert-Jung 1800; Mannheim, Germany) at –25ºC, and stored at –80ºC until staining was performed. For each muscle, the caudal and rostral segments were cut transversely (10 µm). The cross sections were stained with routine hematoxylin and eosin (H&E) staining to examine muscle structure and myofiber morphology. The middle muscle segment was cut sagittally (60 µm). The sagittal sections were stained using histochemical and immunohistochemical methods to document nerve regeneration, axonal sprouts, and reinnervation of the denervated MEPs in the target muscle.

For comparison, five right SM muscles denervated for 3 months by resecting a 5-mm segment of its innervating nerve in 5 additional rats were also prepared as described above and processed together with the experimental muscles.

2.5. Assessments of nerve regeneration and muscle reinnervation

Neurofilament (NF) Immunostaining. Some sagittal sections were immunostained with a monoclonal antibody against phosphorylated NF (SMI-31, Covance Research Products, Berkeley, CA) as a marker for all axons as described in our previous publications [34]. Briefly, the sections were: (1) treated in PBS containing 0.3% Triton and 2% BSA for 30 minutes; (2) incubated with primary antibody SMI-31 (dilution 1:800) in PBS containing 0.03% Triton at 4ºC overnight; (3) incubated for 2 hours with the biotinylated secondary antibody (anti-mouse, 1:1000, Vector, Burlingame, CA); (4) treated with avidin-biotin complex method with a Vectastain ABC kit (1:1000 ABC Elite, Vector); and (5) treated with diaminobenzidine-nickel as chromogen to visualize peroxidase labeling. Control sections were stained as described except that the incubation with the primary antibody was omitted.

Assessment of NF-immunoreactive (NF-ir) axons. The stained sections were examined under a Zeiss photomicroscope (Axiophot-2; Carl Zeiss, Gottingen, Germany) and photographed using a digital camera

(Spot-32; Diagnostic Instruments, Keene, NH). The muscle sections immunostained for NF permit to show how the regenerating axons from the implanted SM nerve reinnervate the denervated SM and quantify the density and spatial distribution of the regenerating axons in the target muscle. The extent of axonal regeneration and muscle reinnervation was evaluated by quantifying the NF-ir axons within the treated muscles as described in our previous publications [29,31]. The intramuscular axonal density was assessed by estimating the number of the NF-ir axons and the area fraction of the axons within a section area (1.0-mm^2). For a give muscle, three sections stained for NF were selected at different spatial levels to count NF-ir axons. For each section, six microscopic fields with NF-ir axons were identified and photographed in an order from ventral to dorsal aspect of the muscle. Areas with NF-positive staining were outlined, measured with public domain ImageJ software (v. 1.45s; NIH, Bethesda, Maryland), and the relation between the operated and control muscles were calculated both for the number and the area fraction of the NF-labeled axons.

2.6. Statistical analysis

Muscle weights, force values, and the number and the area fraction of NF-ir axons of the operated and unoperated SM muscles in each rat were computed. The variables of the reinnervated SM muscles were expressed as the percentages of the values of the contralateral control muscles. All data were presented as mean ± SD. The Student *t* test (paired, 2 tailed) was used to compare differences in the mean muscle force, mean muscle weight, and mean number and area fraction of NF-ir axons between operated and unoperated SM muscles. A difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. Muscle force recovery

At the end of experiments, all rats were subjected to measurement of maximum tetanic tension in the DNI-NMZ reinnervated and contralateral control SM muscles. In one of the rats the nerve on the operated side was damaged during muscle force recording. Muscle force data were successfully collected from both sides

in remaining 14 rats. The degree of functional recovery of the reinnervated SM muscle was determined compared with that of the contralateral control muscle in each of 14 rats. The DNI-NMZ reinnervated SM muscles produced 63.6% of the maximal tetanic tension of the contralateral control muscles (Fig. 3). Averaged maximal muscle force at the operated side was 0.763 N, whereas 1.200 N at the contralateral control side. The difference between these averages (0.437 N) was statistically significant ($P = 0.0013$, $t = 4.08$, $df = 13$, 2 sided t-test). The average rate (calculated between operated and control muscles) was 0.640. This rate was statistically different from 1 ($P = 0.0022$, $t = 3.80$, 2 sided single sample t-test).

3.2. Muscle mass, structure, and myofiber morphology

Three months after muscle reinnervation with DNI-NMZ technique, gross muscle examination showed that the mass of the DNI-NMZ reinnervated muscles was smaller than that of the contralateral muscle (Fig. 4B). The mean value of the wet weight of the reinnervated SM muscles (0.251 g) was smaller compared with that of the control muscles (0.352 g) but larger than that of the denervated SM muscles (0.199 g). Specifically, DNI-NMZ reinnervated SM muscles weighed 71% of the weight of contralateral control muscles (Table 1). The mean percent of wet weight (in relation to normal contralateral side) of DNI-NMZ reinnervated SM muscles (71%) was much higher than that of the denervated SM muscles (44%; $t = 4.72$, $DF = 30$, $P < 0.0001$). Histologically stained cross sections showed that the DNI-NMZ reinnervated muscles exhibited slight-to-moderate fiber atrophy as compared with the controls (Fig. 4C and D). In contrast, denervation resulted in significant myofiber atrophy as indicated by a reduction in fiber size and an increase in connective tissue (data not shown).

3.3. Nerve regeneration and muscle reinnervation

Three months after surgery, muscle sections immunostained for NF showed that DNI-NMZ technique resulted in abundant nerve regeneration. Specifically, the muscle sections from DNI-NMZ reinnervated SM exhibited abundance of axons. The regenerating axons from the implanted SM nerve supplied the denervated

native motor zone within the target muscle (Fig. 5A). The density of the regenerating axons in the reinnervated muscles as indicated by the number and the area fraction of labeled axons were summarized in Table 2. The mean number and area of the NF-ir axons in the reinnervated SM muscles (Fig. 5A and 5A') was computed to be 62% and 51% of the contralateral control muscles (Fig. 5B and 5B'), respectively.

4. Discussion

To our knowledge, this is the first study to explore whether direct nerve implantation into the native motor zone of the denervated muscle could restore better motor function in a rat model. There are several key findings. First, DNI-NMZ resulted in satisfactory functional recovery as indicated by muscle force measurement. Specifically, DNI-NMZ reinnervated SM muscles produced 64% of the maximal tetanic tension of the contralateral control muscles 3 months after surgery. Second, the muscle mass (71% of the control) and myofiber morphology of the DNI-NMZ reinnervated muscles were preserved well. Finally, reinnervated muscles gained abundant regenerating axons as indicated by the mean number (64% of the control) and area (51% of the control) of the NF-ir axons in the target muscles. The encouraging results from this study pave the path for our further work on the development of novel surgical methods and therapies targeting the NMZ of the denervated muscle.

The NMZ is generally located in the middle region of a skeletal muscle. It contains its innervating nerve, intramuscular nerve terminals, and a motor endplate band with numerous neuromuscular junctions. We have developed a new reinnervation technique called "nerve-muscle-endplate band grafting (NMEG)" [25] and performed a series of reinnervation experiments using this technique in a rat model [25-27,30,31]. A NMEG pedicle was harvested from the NMZ of the sternohyoid (SH) muscle (donor). The pedicle was composed of 3 components: a block of muscle (6x6x3 mm), an intact donor nerve branch, nerve terminals, and endplates. The NMEG pedicle was immedately implanted into an aneural region in the caudal portion of the ipsilateral experimentally denervated sternomastoid (SM) muscle (recipient). This surgical procedure resulted in better functional recovery (67% of the control) [25] as compared with nerve end-to-end

anastomosis (55% of the control) [28,31]. More recently, we modified the NMEG surgery to achieve optimal outcomes. In the modified NMEG, the pedicle from the donor muscle was implanted into the native motor zoon (NMZ) in the target muscle. The NMEG-NMZ technique resulted in extensive nerve regeneration and optimal functional motor recovery (82% of the control) [17]. The outcomes from the NMEG-NMZ are most likely attributed to such a fact that the regenerating axons from the implanted NMEG could easily reach and reinnervate the denervated endplates in the NMZ of the target muscle. If the NMEG pedicle is implanted into an aneural region in the target muscle as designed in our original NMEG procedure, the regenerating axons from the implanted pedicle will take time to form new endplates. Therefore, our original NMEG resulted in suboptimal functional recovery. These findings suggest that NMZ in a skeletal muscle is an ideal site for muscle reinnervation. This concept also gains support by previous studies which reported that after nerve injury regenerating nerve fibers preferentially grow into and reinnervate the regions of the original endplates [18-22].

DNI into the denervated muscle (neurotization) is an option for muscle reinnervation when nerve anastomosis, nerve transfer, or a graft is not available [35-46]. However, the effect of DNI on muscle functionality is not fully understood [35-38]. As reported, functional recovery may not be achieved for a long period after DNI surgery [38,43]. Preclinical and clinical studies have demonstrated that the outcome of DNI is associated with the chronicity of denervation [38,44,45], regeneration distance [42,43,45], distance between the nerve implantation site and the native motor zone [40], and surgical techniques [43,46,47]. Using DNI model, some investigators observed preferential reinnervation of the native endplates in the target muscle by abundant regenerating axons and sprouts [23,24]. However, little is known whether DNI-NMZ reinnervation results in satisfactory functional recovery. This study was designed to test our hypothesis that better outcomes could be achieved by implanting a nerve stump into the NMZ within the target muscle. Our results showed that DNI-NMZ resulted in better functional recovery (64% of the control) as compared with that reported in other studies (50%), in which DNI was not specifically performed in the NMZ of the denervated muscles [35,36,45]. The encouraging results from this study should be attributed to such a fact

that DNI-NMZ shortens the distance between the nerve implantation site and the denervated endplates in the target muscle. Therefore, the regenerating axons from the implanted nerve are able to reach and reinnervate the denervated endplates. Studies have demonstrated that after nerve injury regenerating axons preferentially form synapses at original synaptic sites [48-52] and that functional motor recovery is primarily determined by the time to motor endplate reinnervation and the absolute number of regenerated motor axons that reach target [53].

This study showed that DNI-NMZ has the potential for muscle reinnervation. Our ongoing work is to improve outcomes by modifying surgical procedures and using additional approaches that accelerate nerve regeneration. For example, the donor nerve may be divided into two or more fascicles before implantation. Direct implantation of the divided nerve fascicles into the target muscle has shown to enhance end results [11,24]. This procedure may be applied to DNI-NMZ to promote the efficacy of this reinnervation technique. To accelerate nerve regeneration, we will use a very brief 1-hour period of low-frequency (20 Hz) continuous electrical stimulation of the transected and repaired proximal nerve stump at the time of operation as described [54-56]. In addition, nerve growth factor and other exogenous neurotrophic factors will be used with DNI-NMZ technique to enhance axon regeneration [57,58]. We believe that these approaches would promote the outcomes of the DNI-NMZ technique.

Conclusions

In summary, DNI-NMZ technique appears to be a promising reconstructive option for denervated muscles. For optimal outcome, further studies are needed to promote the efficacy of this technique. DNI-NMZ has the potential for functional motor restoration of denervated muscles in certain conditions.

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In conducting research using animals, the investigators adhere to the laws of the United States and regulations of the Department of Agriculture. This protocol was approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of rats.

Disclosure

The authors declare no conflict of interests. The authors report no proprietary or commercial interest in any product mentioned or concepts discussed in the article.

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Figure Legends

Fig. 1. – Native motor zone (NMZ) of the sternohyoid (SM) muscle and surgical procedures for reinnervation of the experimentally denervated SM with direct nerve implantation (DNI) in the rat. (**A**) NMZ of the rat right SM in the middle portion of the muscle between dashed lines in fresh (left), Sihler's stained (middle), and acetylcholinesterase (AChE, right) stained muscles. Note that SM nerve enters the NMZ on the lateral margin of the muscle (arrow in the left image). Sihler's stain, a wholemount nerve staining technique showed that the SM nerve gives off numerous intramuscular twigs and nerve terminals within the NMZ of the muscle. Wholemount AChE staining showed that a motor endplate band with numerous neuromuscular

junctions (arrow) is located within the NMZ of the muscle. (**B-D**) Photographs from a rat during surgery, showing procedures for reinnervation of the right denervated SM muscle with DNI. Entrance point (arrow) of the SM nerve into the muscle was visualized after lateral retraction of muscle tissue (**B**). The SM nerve was transected at the motor point (arrow) and isolated from surrounding tissues (**C**). The proximal stump of the severed SM nerve was buried into a small muscle slit made in the NMZ of the denervated SM muscle, and secured in position with an epineurial suture of 10-0 nylon (**D**). The oblique dashed line in **B-D** indicates the midline. H, hyoid bone; L, left; R, right; S, sternum.

Fig. 2. – Schematic illustration of the force data acquisition system that provides electrical stimulation and records muscle force. A Dell laptop with user-written software in LabVIEW 8.2 is used for controlling the experiment. Surgically detached rostral tendon of the sternomastoid (SM) muscle is attached to the lever of servomotor, which controls muscle stretch and measures muscle force. Electric stimulation with parameters controlled by LabVIEW software is generated by the Multifunctional board 6251 and delivered to the SM nerve. Data are analyzed offline with DIAdem 11.0 software.

Fig. 3. – Muscle force as a function of stimulation current in operated and control SM muscles. The passive tension was set at a moderate level (0.08 N). Stimulation was made with a 0.2-second train of 0.2-millisecond-wide biphasic pulses at frequency of 200 Hz. The lower graph (**B**) shows in expanded scale the early portion of the upper graph (**A**). Note that operated SM muscle (shown in red) when compared to control muscle at the opposite side (shown in black) has larger stimulation threshold (0.02mA vs 0.0075mA), reach the level of maximal force with larger stimulation current (0.3mA vs 0.05mA), and has smaller maximal force. Maximal muscle force level was calculated as the average muscle force to stimulation currents from 0.6-1mA. Average maximal muscle force level at the operated side (0.76 N) was 63.6% of muscle force at the control side (1.20 N). Vertical bars represent the standard deviation of the mean.

Fig. 4. – Photographs of a pair of sternomastoid (SM) muscles removed from a rat 3 months after direct nerve implantation, showing the difference in the morphology of the reinnervated and contralateral control muscles. (**A**) An image taken before removal of the SM muscles. The arrow indicates the implanted SM nerve. The dashed line is the midline. H, hyoid bone; S, sternum. (**B**) A photograph taken after removal of both SM muscles. Note that the mass of the right (R) operated SM muscle (0.312 g) was smaller (moderate atrophy) as compared with that of the left (L) control muscle (0.390 g). (**C**-**D**) Photomicrographs of hematoxylin and eosin-stained cross sections from the SM muscles in B. Note that 3 months after surgery the right SM muscle reinnervated with DNI (**C**) exhibited mild to moderate myofiber atrophy. Initial magnification x 200 for C and D.

Fig. 5. – Images of sagittal sections immunostained for neurofilament (NF) from the operated and unoperated stenomastoid (SM) muscles of the rat #15. (**A**) Six microscopic fields in a stained section from the right operated SM (first column), showing the NF-positive axons (dark staining) in the native motor zone of the reinnervated muscle. Magnification x200. (**A'**) The images in the first column were converted to black and white (second column) by use of public domain ImageJ software (v. 1.45s; NIH, Bethesda, Maryland) to calculate the number and % area of staining in each section (mean axon count: 533; 56% of the control; mean area: 0.636; 43% of the control). (**B**) Six microscopic fields in a stained section from the left unoperated SM (third column), demonstrating the NF-positive axons in the native motor zone of the muscle. Magnification x200. (**B'**) The images in the third column were converted to black and white (fourth column) to compute the number and area of the stained axons (mean axon count: 974. mean area: 1.479).

Table 1

TABLE 1. Wet Muscle Weight Measurement for Imm-DNI Group

Animal No.	Body Weight (g)	Right Reinnervated SM (g)	Left Intact SM (g)
1	353	0.234	0.352
2	347	0.250	0.392
3	343	0.220	0.312
4	340	0.286	0.382
5	327	0.275	0.298
6	322	0.300	0.417
7	355	0.277	0.355
8	328	0.212	0.390
9	316	0.160	0.282
10	332	0.229	0.310
11	355	0.210	0.361
12	378	0.253	0.356
13	349	0.299	0.341
14	407	0.312	0.390
15	380	0.255	0.336
Total	5,232	3.772	5.274
Average	349	0.251	0.352
Ratio, %		71	100

SM, sternomastoid muscle

Table 2

TABLE 2. Comparison of Count and %Area of Neurofilament-Positive Axons between Right Operated and Left Control Sternomastoid (SM) Muscles in Rats (n=15)

Animal	Right SM		Left SM		Ratio (R/L)	
No.	Count	%Area	Count	%Area	Count	%Area
1	511	0.412	1640	1.336	0.312	0.308
2	668	0.430	678	0.626	0.985	0.687
3	420	0.552	1160	1.145	0.362	0.482
4	300	0.456	720	0.566	0.417	0.806
5	473	0.762	510	0.908	0.927	0.839
6	441	0.560	1074	1.358	0.411	0.412
7	312	0.370	600	1.100	0.520	0.336
8	340	0.247	649	0.931	0.524	0.265
9	624	0.623	861	1.295	0.725	0.481
10	671	0.660	752	1.303	0.892	0.507
11	499	0.640	623	1.557	0.801	0.411
12	435	0.511	814	1.496	0.534	0.342
13	319	0.509	893	1.280	0.357	0.398
14	748	0.630	751	0.716	0.996	0.880
15	533	0.636	947	1.479	0.563	0.430
Average	486	0.533	845	1.140	0.622	0.506

Figure 1

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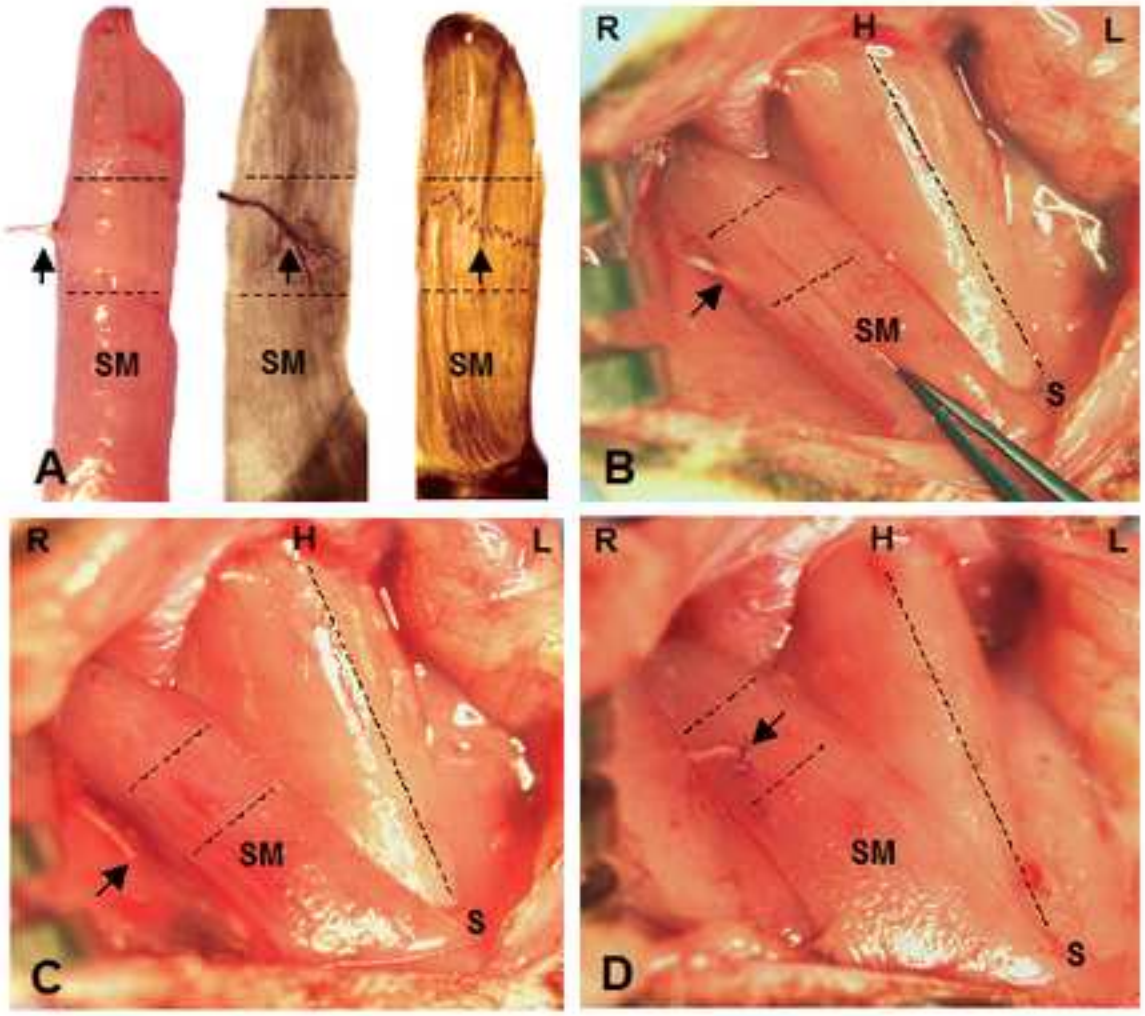

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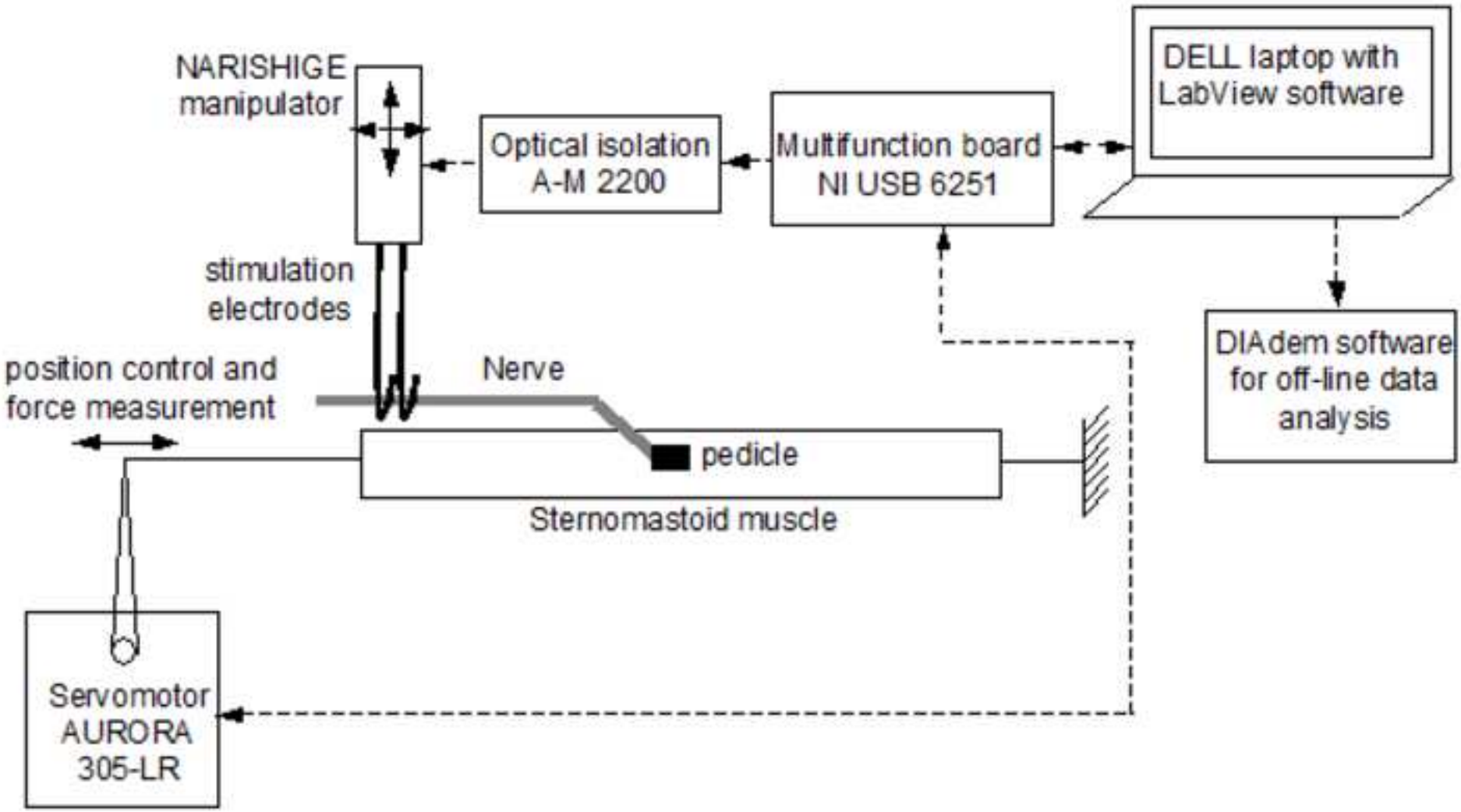

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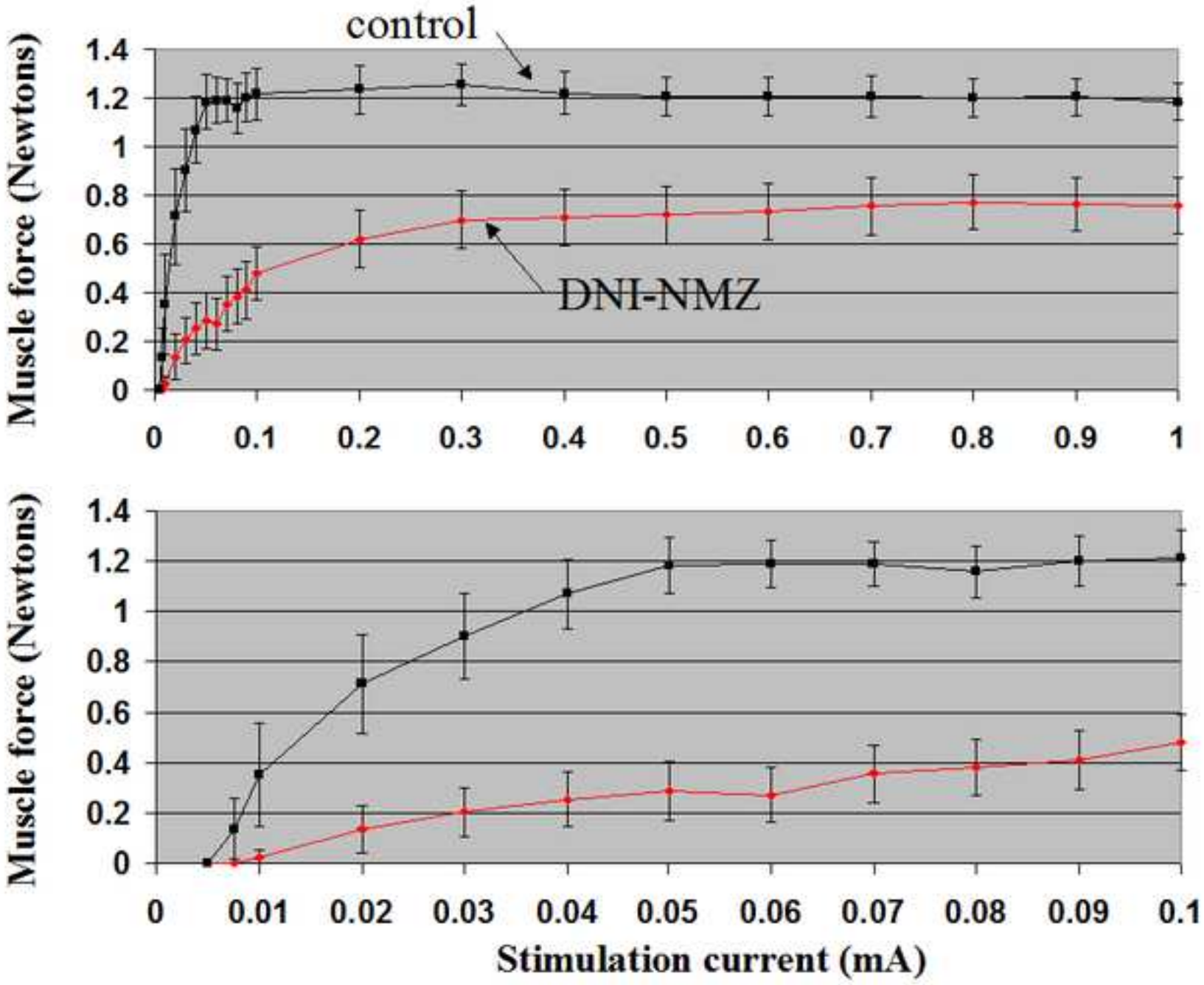

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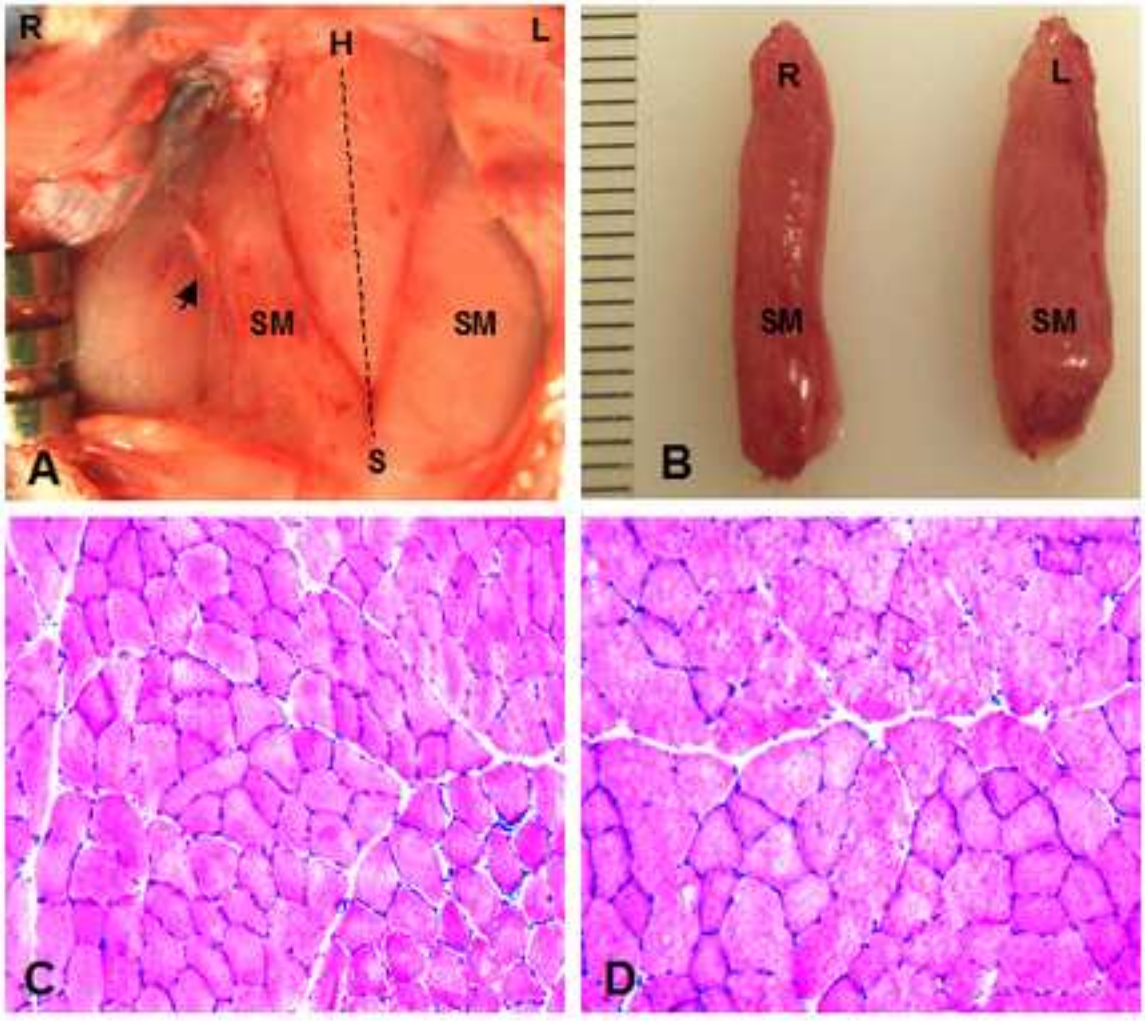

Figure 5

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